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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(71)(72) Applicants and Inventors: CHAUX, Pascal [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). LUITEN, Rosalie [NL/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). DEMOTTE, Nathalie [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). DUFFOUR, Marie–Thérèse [FR/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). LURQUIN, Christophe [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). TRAVERSARI, Catia [IT/BE]; Dipartimento di Biologia e Biotechnologia (DIBIT), Intituto Scientifico H.S. Raffaele, Via Olgettina, 60, I–20132 Milan (IT). STROOBANT, Vincent [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). CORNELIS, Guy, R. [BE/BE]; International Institute of Cell & Molecular Pathology, Microbial Pathogenesis Unit, University of Louvain Medical Faculty, Avenue Hippocrate 74, P.O. Box 74.49, B–1200 Brussels (BE). BOON–FALLEUR, Thierry [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). VAN DER BRUGGEN, Pierre [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE). SCHULTZ, Erwin [DE/BE]; Rue Pere Endore Decroye 104, B–1150 Brussels (BE). WARNIER, Guy [BE/BE]; Ludwig Institute for Cancer Research, UCL 7459, Avenue Hippocrate 74, B–1200 Brussels (BE).

(74) Agents: KOLB, Helga et al.; Hoffmann . Eitle, Arabellastrasse 4, D–81925 München (DE).

(54) Title: TUMOR ANTIGENS AND CTL CLONES ISOLATED BY A NOVEL PROCEDURE

(57) Abstract

The present invention relates to isolation of cytotoxic T lymphocyte (CTL) clones. In particular, the present invention relates to isolated CTL clones that are specific for proteins of the MAGE family. The CTL clones of the present invention have been isolated by successive steps of stimulation and testing of lymphocytes with antigen presenting cells which present antigens derived from different expression systems, e.g., from recombinant *Yersinia*, recombinant *Salmonella*, or recombinant viruses. The present invention further relates to antigenic peptides as well as the peptide/HLA complexes which are recognized by the isolated CTL clones.

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## TUMOR ANTIGENS AND CTL CLONES
## ISOLATED BY A NOVEL PROCEDURE

**FIELD OF INVENTION**

The present invention relates to isolation of
cytotoxic T lymphocyte (CTL) clones.  The CTL clones of
the present invention have been isolated by successive
steps of stimulation and testing of lymphocytes with
antigen presenting cells which present antigens derived
from different expression systems, e.g., from recombinant
*Yersinia*, recombinant *Salmonella*, or recombinant viruses.
The present invention further relates to isolated CTL
clones that are specific for proteins of the MAGE family.
Antigenic peptides as well as the peptide/HLA complexes
which are recognized by the isolated CTL clones are also
provided.


**BACKGROUND**

An important facet of the immune response in a
mammalian subject is the recognition by T cells of the
complexes of the cell surface molecules, i.e., the
complexes of peptides and HLA (human leukocyte antigens)
or MHC (major histocompatibility complexes) molecules.
These peptides are derived from larger molecules which
are processed by the cells which also present the HLA/MHC
molecules.  See in this regard, Male et al., *Advanced
Immunology* (J.P. Lipincott Company, 1987), especially
chapters 6-10.  The interaction between T cell and
HLA/peptide complexes is restricted, requiring a T cell
specific for a particular combination of an HLA molecule
and a peptide.  If a specific T cell is not present,
there is no T cell response even if its partner complex
is present.  Similarly, there is no response if the

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specific complex is absent, but the T cell is present. This mechanism is involved in the immune system response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities.

Most progressively growing neoplastic cells express potentially immunogenic tumor-associated antigens (TAAs), also called tumor rejection antigens (TRAs). A number of genes have been identified that encode tumor rejection antigen precursors (or TRAPs), which are processed into TRAs in tumor cells. Such TRAP-encoding genes include members of the MAGE family, the BAGE family, the DAGE/PRAME family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp 100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as praise, mutated tumor suppressor genes such as p53, tumor associated viral antigens such as HPV16 E7. See, e.g., review by Van den Eynde and van der Bruggen (1997) in *Curr. Opin. Immunol.* 9:684-693, Sahin et al. (1997) in *Curr. Opin. Immunol.* 9:709-716, and Shawler et al. (1997) *Advances in Pharmacology 40*: 309-337, Academic Press, Inc., San Diego, California.

TRAs, like other antigenic epitopes, are presented at the surface of tumor cells by MHC molecules and have been shown to induce a CTL response *in vivo* and *in vitro*. See, for example, van der Bruggen et al. (1991) *Science 254*: 1643-1647. However, such TRA-expressing tumor cells do not provoke reliable anti-tumor immune responses *in vivo* that are capable of controlling the growth of malignant cells. Boon et al. (1992) *Cancer Surveys 13*: 23-37; T. Boon (1993) *Int. J. Cancer 54*: 177-180; T. Boon (1992) *Advances Cancer Res. 58*: 177-209. Thus, generation of CTL clones that recognize specific TRAs provides a powerful tool for

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tumor therapeutics.  The identification of TRAs also allows the design of recombinant vaccines for the treatment of various pathological conditions.

The present invention provides a novel procedure for isolating CTL clones.  By following such procedure, novel CTL clones have been isolated that recognize specific antigenic peptides of proteins, preferably of the MAGE family.  The MHC molecules presenting these peptides have been identified as well.

## SUMMARY OF THE INVENTION

One embodiment of the present invention provides methods for isolating CTL clones from a blood sample.

The methods of the present invention include successive steps of stimulating and testing lymphocytes with antigen presenting cells.  Such methods, by employing different antigen presenting cells at different steps, significantly reduce non-specific CTL activities generated in the procedure and permit more efficient isolation of CTL clones.

Antigen presenting cells which are used in the methods of the present invention can differ in cell type and/or in the expression system from which the antigen to be presented is derived.  Cells which can be employed as antigen presenting cells in the present methods include professional and facultative antigen presenting cells.  A preferred antigen presenting cell is an autologous dendritic cell, an autologous B cell transformed with EBV, or an activated T cell.

Antigen presenting cells can be modified by a variety of ways to effect the expression of an antigen of interest at the cell surface, preferably, by infection

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with a recombinant *Yersinia*, recombinant *Salmonella*, or recombinant viruses. Preferred recombinant viruses include vaccinia, canarypox virus, other pox viruses, adenovirus, herpes simplex virus, and retrovirus.

The protein against which CTL clones are generated can be a tumor associated protein, an antigenic protein of a pathogen, or the like. Preferably, the protein is a member of the MAGE family, in particular, MAGE-A1, MAGE-A3 and MAGE-A4.

In another embodiment, the present invention contemplates CTL clones isolated by using the methods of the present invention.

In a preferred embodiment, the present invention provides isolated CTL clones that are specific for peptide/HLA complexes SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL (SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL (SEQ ID NO: 57)/HLA-B7, respectively.

In a more preferred embodiment, the present invention provides isolated CTL clones LB1137 462/F3.2, LB1801 456/H7.11, LB1118 466/D3.31, LB 1801 456/H8.33, LB1137 H4.13, LB1841 526/F7.1 and LB1803 483/G8.4.

Furthermore, the present invention provides methods of identifying antigenic peptide epitopes of a protein by using CTL clones isolated following the methods of present invention.

In still another embodiment, the present invention provides newly isolated antigenic peptides, DPARYEFLW (MAGE-A1 258-266) (SEQ ID NO: 42), GVYDGREHTV (MAGE-A4 230-239) (SEQ ID NO: 44), SAFPTTINF(SEQ ID NO: 47) (MAGE-A1 62-70), EVYDGREHSA(SEQ ID NO: 48) (MAGE-A1

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222-231), AELVHFLLL (SEQ ID NO: 55) (MAGE-A3 114-122),
RVRFFFPSL (SEQ ID NO: 57) (MAGE-A1 289-297). Nucleic
acid sequences encoding such peptides are also
contemplated.

In another embodiment, the present invention
provides isolated peptide/HLA complexes, peptide
SAYGEPRKL (SEQ ID NO: 2) complexed with HLA-Cw3, peptide
DPARYEFLW (SEQ ID NO: 42) complexed with HLA-B53, peptide
GVYDGREHTV (SEQ ID NO: 44) complexed with HLA-A2, peptide
SAFPTTINF (SEQ ID NO: 47) complexed with HLA-Cw2,
EVYDGREHSA (SEQ ID NO: 48) complexed with HLA-A28,
AELVHFLLL (SEQ ID NO: 55) complexed with HLA-B40, and
RVRFFFPSL (SEQ ID NO: 57) complexed with HLA-B7.

In another embodiment, cells expressing any of
these peptide/HLA complexes are contemplated.

Still another embodiment of the invention
provides pharmaceutical compositions which include any
one of the isolated CTL clones, the antigenic peptides,
the peptide/HLA complexes, and cells expressing the
peptide/HLA complexes of the present invention.

In a further aspect, the present invention
provides methods useful for diagnosing and treating
various pathological conditions.

One embodiment of the present invention
provides methods of diagnosing in a subject, a
pathological condition characterized by an abnormal
expression of a peptide/HLA complex, by detecting the
presence of cells abnormally expressing such complex in
the subject.

Another embodiment of the present invention
provides methods of detecting in a subject, the presence
of cells abnormally expressing a peptide/HLA complex of
the present invention by using an isolated CTL clone of

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the present invention which specifically recognizes such complex.

One embodiment of the present invention provides methods of diagnosing in a subject, a pathological condition characterized by an abnormal expression of a peptide/HLA complex, by detecting an increased frequency of CTL cells specific for such complex.

Another embodiment of the present invention provides methods of detecting in a subject the presence, of CTL cells specific for a peptide/HLA complex of the present invention by using an antigen presenting cell expressing such complex at the cell surface.

In still another embodiment, the present invention provides methods of treating a subject of a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention by administering to the subject, a therapeutically effective amount of cells of a CTL clone specific for such complex.

Another embodiment provides methods of treating a subject of a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention, by administering to the subject a therapeutically effective amount of the peptide.

Still another embodiment provides methods of treating a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention, by obtaining antigen presenting cells from the subject, modifying such cells to effect a presentation of the peptide/HLA complex at the cell surface, and then reperfusing such "loaded" cells into the subject.

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## BRIEF DESCRIPTION OF FIGURES

**Figure 1** illustrates the plasmid map of the expression vector pMS111-MAGE-A1 (YopE$_{130}$-MAGE-A1).

**Figure 2** (A) depicts the procedure for stimulating CTL 82/30 with EBV-transformed human B cells (HLA-A1) mixed with recombinant *Yersinia*; (B) depicts the quantitation of IFN-released by activated CTLs.

**Figure 3** depicts the specific recognition by CTL clone LB1137 462/F3.2 of a MAGE-A1 antigenic peptide presented by HLA-Cw3.

**Figure 4** depicts the specific recognition by CTL clone LB1801 456/H7.11 of a MAGE-A1 antigenic peptide presented by HLA-B53.

**Figure 5** depicts the specific recognition by CTL clone LB1137 H4.13 of a MAGE-A4 antigenic peptide presented by HLA-A2.

**Figure 6** depicts the MAGE-A4 nucleotide sequences (SEQ ID NO: 49) and the primers used in PCR as described in Example 9.

**Figure 7** depicts a MAGE-A1 peptide presented by HLA-Cw2 to CTL clone LB1118 466/D3.31.

**7A.** Lysis by CTL clone LB1118 466/D3.31 of autologous EBV-B cells infected with vaccinia-MAGE-A1. Target cells were infected for 2 hours at an MOI of 20, $^{51}$Cr-labeled, and incubated with CTL clone LB1118 466/D3.31 for 4 hours. Targets infected with the parental vaccinia were used as a negative control.

**7B.** Stimulation of CTL clone LB1118 466/D3.31 by COS-7 cells that were transiently transfected with a MAGE-A1 cDNA and a cDNA encoding HLA-Cw2. One day after transfection, 1,500 CTL clone LB1118 466/D3.31 were added

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into microwells containing $1.5 \times 10^4$ transfected COS-7 cells. TNF production was estimated after overnight coculture by testing the toxicity of the supernatants for the TNF-sensitive cells of WEHI-164 clone 13.

**7C**. Lysis by CTL clone LB1118 466/D3.31 of autologous EBV-B cells incubated with synthetic peptide SAFPTTINF(SEQ ID NO: 47) (MAGE-A162-70). Targets were 52Cr-labeled and incubated for 4 hours with the CTL, at an effector-to-target ratio of 5:1, in the presence of the peptide at the concentrations indicated.

**7D**. Lysis of HLA-Cw2 tumor cell lines by CTL clone LB1118 466/D3.31. Target cells were 51Cr-labeled and incubated for 4 hours with CTL clone LB1118 466/D3.31 at various effector-to-target ratios.

**Figure 8** depicts a MAGE-A1 peptide presented by HLA-A28 to CTL clone LB1801 456/H8.33.

**8A**. Lysis by CTL clone LB1801 456/H8.33 of autologous EBV-B cells infected with vaccinia-MAGE-A1. Target cells were infected for 2 hours at an MOI of 20, 51Cr labeled, and incubated with CTL clone LB1801 456/H8.33 for 4 hours. Targets infected with the parental vaccinia were used as a negative control.

**8B**. Stimulation of CTL clone LB1801 456/H8.33 by COS-7 cells transiently transfected with a MAGE-A1 cDNA and a cDNA encoding HLA-A28. One day after transfection, 1,500 CTL clone LB1801 456/H8.33 were added into microwells containing $1.5 \times 10^4$ transfected COS-7 cells. TNF production was estimated after overnight coculture by testing the toxicity of the supernatants for the TNF-sensitive cells of WEHI-164 clone 13.

**8C**. Lysis by CTL clone LB1801 456/H8.33 of autologous EBV-B cells incubated with synthetic peptide

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EVYDGREHSA(SEQ ID NO: 48) (MAGE-A1 $_{222-231}$). Targets were $^{51}$Cr-labeled and incubated for 4 hours with CTL clone LB1801 456/H8.33, at an effector-to-target ratio of 5:1, in the presence of the peptide a the concentrations indicated.

      **8D.** Lysis of HLA-A28 melanoma line by CTL clone LB1801 456/H8.33. Target cells were $^{51}$Cr-labeled and incubated for 4 hours with CTL clone LB1801 456/H8.33 at various effector-to-target ratios.

      **Figure 9.** A MAGE-A3 peptide is presented to CTL clone LB1841 526/F7.1 by HLA-B40.

      **9A.** Lysis by CTL clone LB1841 526/F7.1 of autologous EBV-B cells infected with vaccinia-MAGE-A3.

      **9B.** Stimulation of CTL clone LB1841 526/F7.1 by COS-7 cells transiently transfected with a MAGE-A3 cDNA and a cDNA encoding an HLA molecule as indicated.

      **9C.** Lysis by CTL clone LB1841 526/F7.1 of autologous EBV-B cells incubated with synthetic peptide AELVHFLLL (SEQ ID NO: 55) (MAGE-A3 $_{114-122}$).

      **9D.** Lysis of HLA-B40 melanoma cells by CTL clone LB1841 526/F7.1.

      **Figure 10.** A MAGE-A1 peptide is presented to CTL clone LB1803 483/G8.4 by HLA-B7.

      **10A.** Lysis by CTL clone LB1803 483/G8.4 of autologous EBV-B cells infected with vaccinia-MAGE-A1.

      **10B.** Stimulation of CTL clone LB1803 483/G8.4 by COS cells transiently transfected with a MAGE-A1 cDNA and a cDNA encoding an HLA molecule as indicated.

      **10C.** Lysis by CTL clone LB1803 483/G8.4 of autologous EBV-B cells incubated with synthetic peptide RVRFFFPSL (SEQ ID NO: 57) (MAGE-A1 $_{289-297}$).

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**10D.**  Lysis of HLA-B7 melanoma cells by CTL clone LB1803 483/G8.4.

## DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention provides novel methods for isolating CTL clones.  The present methods include successive steps of stimulating and testing lymphocytes by using different antigen presenting cells at different steps.

The procedure to develop specific CTL clones *in vitro* has been described.  Briefly, a blood sample containing T-cell precursors is taken from a mammal.  PBLs are purified from such blood sample and are incubated with stimulator cells which express antigenic peptides complexed with the appropriate MHC molecule.  Stimulator cells can be tumor cells (see, e.g., the United States Patent No. 5,342,774, Knuth et al. (*Proc. Natl. Acad. Sci. USA* 86:  2804-2808, 1989) and Van Den Eynde et al. (*Int. J. Cancer* 44:  634-640, 1989), or antigen presenting cells pulsed with defined peptides.  Additional components, e.g., allogeneic feeder cells and cytokines, can be added into the incubation mixture.  CTLs specific for antigens expressed at the surface of the stimulator cells will proliferate, and thus, will be enriched in the cell population as a result of the stimulation.  CTL clones can be subsequently isolated by, e.g., limiting dilution.  However, the approach using antigen presenting cells pulsed with defined peptides as stimulator cells, have sometimes generated CTLs that are unable to recognize the relevant tumor cells.

The present inventors have found that efficient isolation of CTL clones can be achieved by successive steps of stimulating and testing T cell precursors, using

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different antigen presenting cells at different steps. The present methods of isolating CTL clones permit significant reduction of CTL activities generated toward non-specific molecules, e.g., molecules expressed from the backbone sequence of an expression vector.

By "different antigen presenting cells" it means that the antigen presenting cells may differ in cell type or in the expression system from which an antigen of interest being presented is derived.

"Antigen presenting cells" as referred herein, express at least one class I or class II MHC determinant and may include those cells which are known as professional antigen-presenting cells such as macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used according to the present invention. Examples of facultative antigen-presenting cells include activated T cells, astrocytes, follicular cells, endothelium and fibroblasts. As used herein, "antigen-presenting cells" encompass both professional and facultative types of antigen-presenting cells.

The antigen presenting cells can be isolated from tissue or blood samples (containing peripheral blood mononuclear cells) obtained from a mammal such as human. Cell lines established from such samples may also be used. Procedures for establishing cell lines are well known in the art. Certain cell lines may be obtained directly from the American Type Culture Collection, 12301

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Parklawn Drive, Rockville, Maryland, 20852-1776. Both normal and malignant cells can be employed.

Preferably, the MHC determinants expressed by the antigen presenting cells are compatible with those expressed by the mammal from which the sample containing T cell precursors is taken. More preferably, autologous antigen presenting cells or cell lines established therefrom are employed. Non-autologous cells may be used as long as the MHC determinants expressed by such cells are compatible, either naturally, by way of transfection or other means that are appropriate. One skilled in the art is also familiar with the methods for determining whether the MHC molecules expressed by an antigen presenting cell are compatible with those of the mammal subject involved, such as well known HLA-typing procedures. See general teachings by Coligan et al. (1994) *Current Protocols in Immunology* John Wiley & Sons Inc: New York, New York.

Preferred antigen presenting cells are autologous dendritic cells, autologous B cells transformed with EBV, and autologous T cell activated by PHA.

Further, according to the present invention, antigen presenting cells used in the present methods can also differ in the expression system from which an antigen of interest is derived. More specifically, the antigen presenting cells can be modified in various ways to effect the expression of an antigen at the cell surface. For example, an antigen presenting cell can be infected with a recombinant *Yersinia*, a recombinant *Salmonella*, or a recombinant virus. In each case, the recombinant microorganism encodes a protein from which the peptide antigen presented is derived.

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The protein expressed from any of these
expression systems is processed in the antigen presenting
cells into small peptides, which are then complexed with
the appropriate MHC molecules and presented at the cell
surface.  In the present invention, peptides that are
complexed with MHC molecules and presented at the cell
surface are also referred to as "antigens".

The term "*Yersinia*" as used herein includes all
species of *Yersinia*, including *Yersinia enterocolitica*,
*Yersinia pseudotuberculosis* and *Yersinia pestis*.  The
term "recombinant *Yersinia*" used herein refers to
*Yersinia* genetically transformed with an expression
vector.  The term "delivery" used herein refers to the
transportation of a protein from a *Yersinia* to an antigen
presenting cell, including the steps of expressing the
protein in the *Yersinia*, secreting the expressed
protein(s) from such *Yersinia* and translocating the
secreted protein(s) by such *Yersinia* into the cytosol of
the antigen presenting cell.

According to the present invention, preferred
*Yersinia* for use in expressing and delivering the protein
of interest are mutant *Yersinia* that are deficient in
producing functional effector proteins.

A preferred mutant *Yersinia* strain for use in
expressing and delivering the protein of interest is a
quintuple-mutant *Yersinia* strain in which all the
effector-encoding genes are mutated such that the
resulting *Yersinia* no longer produce any functional
effector proteins.  Such quintuple-mutant *Yersinia* strain
is designated as *yopEHOMP* for *Y. enterocolitica* or
*yopEHAMJ* for *Y. pseudotuberculosis*.  One example of such
*yopEHOMP* strain is *Y. enterocolitica* MRS40(pABL403).

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An antigenic protein of interest can be cloned into a yersinia expression vector Ffr used in combination with a mutant *Yersinia* for delivery of the protein into antigen presenting cells. In accordance with the present invention, such a vector is characterized by (in the 5' to 3' direction) a promoter, a first nucleic acid sequence encoding a delivery signal, a second nucleic acid sequence fused thereto coding for the protein to be delivered and other sequences that may be appropriate (e.g., a polyadenylation signal).

The promoter of the expression vector is preferably from a *Yersinia* virulon gene. A "*Yersinia* virulon gene" refers to genes on the *Yersinia* pYV plasmid, the expression of which is controlled both by temperature and by contact with a target cell. See review by Cornelis et al. (1997). Such genes include genes coding for elements of the secretion machinery (the *Ysc* genes), genes coding for translocators (*YopB*, *YopD*, and *LcrV*), genes coding for the control elements (*YopN* and *LcrG*), and genes coding for effectors (*YopE*, *YopH*, *YopO/YpkA*, *YopM* and *YopP/YopJ*). Preferably, the promoter is from an effector-encoding gene selected from any one of *YopE*, *YopH*, *YopO/YpkA*, *YopM* and *YopP/YopJ*. More preferably, the promoter is from *YopE*.

Further, in accordance with the present invention, a first DNA sequence coding for a delivery signal is operably linked to the promoter. "A delivery signal", as described hereinabove, refers to a polypeptide which can be recognized by the secretion and translocation system of *Yersinia* and therefore directs the secretion and translocation of a protein into a an antigen presenting cell. Such polypeptide is from an effector protein including YopE, YopH, YopO/YpkA, YopM,

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and YopP/YopJ, and preferably, YopE. More preferably, the effector protein is YopE of *Yersinia enterocolitica*.

One skilled in the art is familiar with the methods for identifying the polypeptide sequences of an effector protein that are capable of delivering a protein. For example, one such method is described by Sory et al. (1994). Examples of such delivery signal polypeptides include from *Y. enterocolitica*: $YopE_{130}$ (the N-terminal 130 amino acids of YopE), $YopE_{50}$, $YopM_{100}$ and $YopH_{71}$.

The yersinia expression vectors may be transformed into *Yersinia* by a number of known methods which include, but are not limited to, electroporation, calcium phosphate mediated transformation, conjugation, or combinations thereof. For example, a vector can be transformed into a first bacteria strain by a standard electroporation procedure. Subsequently, such a vector can be transferred from the first bacteria strain into *Yersinia* by conjugation, a process also called "mobilization". *Yersinia* transformant (i.e., *Yersinia* having taken up the vector) may be selected, e.g., with antibiotics. These techniques are well known in the art. See, for example, Sory et al. (1994).

The delivery of a protein from a recombinant *Yersinia* into the cytosol of an antigen presenting cell can be effected by contacting an antigen presenting cell with a recombinant *Yersinia* under appropriate conditions. Multiple references and techniques are available for those skilled in the art regarding the conditions for inducing the expression and translocation of virulon genes, including the desired temperature, Ca++ concentration, manners in which *Yersinia* and target cells are mixed, and the like. See, for example, Cornelis,

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*Cross talk between Yersinia and eukaryotic cells*, Society
for General Microbiology Symposium, 55; Mocrae, Saunders,
Smyth, Stow (eds), *Molecular aspects of host-pathogen
interactions*, Cambridge University Press, 1997.  The
conditions may vary depending on the type of eukaryotic
cells to be targeted, e.g.:  the conditions for targeting
human epithelial carcinoma Hela cells (Sory et al.
(1994)); the conditions for targeting mouse thymoma or
melanoma cells (Starnbach et al. (1994) *J. Immunol. 153*:
1603); and the conditions for targeting mouse macrophages
(Boland et al. (1996)).  Such variations can be addressed
by those skilled in the art using conventional
techniques.

Those skilled in the art can also use a number
of assays to determine whether the delivery of a fusion
protein is successful.  For example, the fusion protein
may be labeled with an isotope or an immunofluoresceine,
or detected by a immunofluoresceine conjugated antibody,
as disclosed by Rosqvist et al. (1994) *EMBO J. 13*:  964.
The determination can also be based on the enzymatic
activity of the protein being delivered, e.g., the assay
described by Sory et al. (1994).  The determination can
also be based on the antigenicity of the protein being
delivered.  For example, the delivery of a MAGE-A1
protein into EBV-transformed human B cells can be
detected by the recognition of such targeted B cells by
CTL cells specific for MAGE-A1 epitopes.  Such CTL
recognition, in turn, may be detected by a number of
assays including assaying the secretion of IFN-$\gamma$ from the
activated CTLs or $Cr^{51}$ release from lysed target cells.
Methods such as Western-blot analysis using antibodies
specific against the protein being delivered, PCR in situ
hybridization, or ELISPOT (Mabtech AB, Sweden) may also

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be employed for such determination. See, e.g., W. Herr et al. (1997) *J. Immunol. Methods 203*: 141-152 and W. Herr et al. (1996) *J. Immunol. Methods 191*: 131-142.

In accordance with the present invention, the antigenic protein of interest can also be expressed from a recombinant Salmonella. For example, avirulent strains of *Salmonella typhimurium* can be used as antigen delivery vectors. It is known in the art that antigenic epitopes, such as viral epitopes can be successfully delivered to the host cell cytosol by using the type III protein secretion system of *S. typhimurium*. See, e.g., Russmann et al. (1998) 281: 565-568.

In accordance with the present invention, the expression of a protein of interest in the antigen presenting cell can also be effected by infection of the antigen presenting cells with a recombinant virus. In particular, the present invention contemplates recombinant viruses of vaccinia, canarypox virus, other pox viruses, adenovirus, herpes simplex virus, retrovirus and any other viruses that are appropriate.

A preferred strain of vaccinia for use in the present invention is the WR strain (Panicali et al.(1981), *J. Virol.* 37: 1000-1010). The nucleotide sequence coding for the protein of interest can be operably linked to a promoter, such as an vaccinia promoter H6, and inserted into a vaccinia vector, thereby generating a donor plasmid. Vaccinia vectors which can be employed for generating adeno-plasmids are available to those skilled in the art and are described in, e.g., U.S. Patent No. 4,769,330. Recombinant WR strains of vaccinia can be generated by using a donor plasmid via *in vivo* recombination, following well-known procedures.

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See, e.g., Perkins et al., *J. Virol.* 63:   3829-3936
(1989).

A preferred strain of canarypox virus for use
in the present invention is ALVAC (Cox et al. (1993),
*Virology* 195:   845-850).   The nucleotide sequence coding
for the protein of interest can be operably linked to a
promoter, such as an vaccinia promoter H6, and inserted
into an ALVAC vector to create a donor plasmid.   Multiple
ALVAC vectors are available to one skilled in the art and
are described by, e.g., U.S. Patent No. 5,756,106; Cox et
al. (1993) *Virology 195*:   845-850; Tartaglia et al.
(1993) *J. Virology 67*: 2370-2375; and Taylor et al.
(1992) *Virology 187*:   321-328.   Such donor plasmid can be
used to generate recombinant ALVAC viruses via in vivo
recombination.   See, e.g., Cox. et al. (1993); Tartaglia
et al. (1993) and Taylor et al. (1992).

Those skilled in the art can also generate
recombinant adenoviruses for expressing the protein of
interest as described in, e.g., Example 5 hereinafter.

A nucleotide sequence encoding the antigenic
protein of interest can be cloned into the various
expression vectors as described above.   There is no
particular limitation in the protein that can be employed
in the instant methods for isolating CTL clones.

The term "protein" as used herein refers to
naturally occurring proteins as well as artificially
engineered proteins, or parts thereof.   The term "part of
a protein" includes a peptide fragment of a protein that
is of sufficient length to be antigenic.   Preferably,
such a fragment consists of at least 8 or 9 amino acids.
"Artificially engineered proteins" as used herein refer
to non-naturally occurring proteins, e.g., modified forms
of non-naturally occurring proteins, or fusion of two or

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more naturally occurring proteins or parts thereof, which are also referred to as polytopes (in-frame fusion of two or more epitopes) as exemplified by Thompson et al. (1995) in *Proc. Natl. Acad. Sci. USA* 92: 5845-5849.

The present invention contemplates, in particular, tumor associated proteins or pathogen associated antigens.

A "tumor associated protein" refers to a protein that is specifically expressed in tumors or expressed at an abnormal level in tumors relative to normal tissues. Such tumor associated proteins include, but are not limited to, members of the MAGE family, the BAGE family (such as BAGE-1), the DAGE/PRAME family (such as DAGE-1), the GAGE family, the RAGE family (such as RAGE-1), the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as p21ras, mutated tumor suppressor genes such as p53, tumor associated viral antigens (e.g., HPV16 E7), the SSX family, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, HOM-TES-11, RCC-3.1.3, NY-ESO-1, and the SCP family. Members of the MAGE family include, but are not limited to, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A11. Members of the GAGE family include, but are not limited to, GAGE-1, GAGE-6. See, e.g., review by Van den Eynde and van der Bruggen (1997) in *Curr. Opin. Immunol.* 9: 684-693, Sahin et al. (1997) in *Curr. Opin. Immunol.* 9: 709-716, and Shawler et al. (1997). These proteins have been shown to associate with certain tumors such as melanoma, lung cancer, prostate cancer, breast cancer, renal cancer and others.

A number of known antigenic proteins from pathogens are also contemplated by the present invention.

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The pathogens can include viruses, bacteria, parasites
and fungi.  Specific examples of antigenic proteins
characteristic of a pathogen include the influenza virus
nucleoprotein (residues 218-226, as set forth in F. et
al. (1997) *J. Virol. 71*:  2715-2721) antigens from Sendai
virus and lymphocytic choriomeningitis virus (see, An et
al. (1997) *J. Virol. 71*:  2292-2302), the B1 protein of
hepatitis C virus (Bruna-Romero et al. (1997) *Hepatology
25*:  470-477), the virus envelope glycoprotein gp 160 of
HIV (Achour et al. (1996) *J. Virol. 70*:  6741-6750),
amino acids 252-260 or the circumsporozite protein of
*Plasmodium berghei* (Allsopp et al. (1996) *Eur. J.
Immunol. 26*:  1951-1958), the influenza A virus
nucleoprotein (residues 366-374, Nomura et al. (1996) *J.
Immunol. Methods 193*:  4149), the listeriolysin O protein
of *Listeria monocytogenes* (residues 91-99, An et al.
(1996) *Infect. Immun. 64*:  1685-1693), the E6 protein
(residues 131-140, Gao et al. (1995) *J. Immunol. 155*:
5519-5526) and E7 protein (residues 21-28 and 48-55,
Bauer et al. (1995) *Scand. J. Immunol. 42*:  317-323) of
human papillomavirus type 16, the M2 protein of
respiratory syncytial virus (residues 82-90 and 81-95,
Hsu et al. (1995) *Immunology 85*:  347- 350), the herpes
simplex virus type 1 ribonucleotide reductase (see,
Salvucci et al. (1995) *J. Gen. Virol. 69*:  1122-1131) and
the rotavirus VP7 protein (see, Franco et al. (1993) *J.
Gen. Virol. 74*:  2579-2586), *P. falciparum* antigens
(causing malaria) and hepatitis B surface antigen
(Gilbert et al. (1997) *Nature Biotech.* 15: 1280-1283).

     A number of short antigenic peptides can also
be employed in the present invention.  One skilled in the
art can readily determine the length of the fragments

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required to produce immunogenic peptides.  Alternatively,
the skilled artisan can also use coding sequences for
peptides that are known to elicit specific T cell
responses (either CD4^{+} or CD8^{+} T cells), such as
tumor-associated antigenic peptides (TAA, also known as
TRAs for tumor rejection antigens) as disclosed by U.S.
Patent No. 5,462,871, U.S. Patent No. 5,558,995, U.S.
Patent No. 5,554,724, U.S. Patent No. 5,585,461, U.S.
Patent No. 5,591,430, U.S. Patent No. 5,554,506, U.S.
Patent No. 5,487,974, U.S. Patent No. 5,530,096, U.S.
Patent No. 5,519,117.  Examples of TRAs are provided in
Table 1.  See also review by Van den Eynde and van der
Bruggen (1997) and Shawler et al. (1997).  Antigenic
peptides of a pathogen origin can also be used, such as
those disclosed by Gilbert et al. (1997).

**Table 1:  Exemplary Antigens**

| Gene | MHC | Peptide | Position | SEQ ID NO: |
|------|-----|---------|----------|------------|
| MAGE-A1 | HLA-A1 | EADPTGHSY | 161-169 | 1 |
| | HLA-Cw16 | SAYGEPRKL | 230-238 | 2 |
| MAGE-A3 | HLA-A1 | EVDPIGHLY | 168-176 | 3 |
| | HLA-A2 | FLWGPRALV | 271-279 | 4 |
| | HLA-B44 | MEVDPIGHLY | 167-176 | 5 |
| BAGE | HLA-Cw16 | AARAVFLAL | 2-10 | 6 |
| GAGE-1,2 | HLA-Cw16 | YRPRPRRY | 9-16 | 7 |
| RAGE | HLA-B7 | SPSSNRIRNT | 11-20 | 8 |
| GnT-V | HLA-A2 | VLPDVFIRC(V) | 2-10/11 | 9 |
| MUM-1 | HLA-B44 | EEKLIVVLF | exon 2/ intron | 10 |

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| | | | | |
|---|---|---|---|---|
| | | EEKLSVVLF (wild type) | | 11 |
| CDK4 | HLA-A2 | ACDPHSGHFV | 23-32 | 12 |
| | | ARDPHSGHFV (wild type) | | 13 |
| β-catenin | HLA-A24 | SYLDSGIHF | 29-37 | 14 |
| | | SYLDSGIHS (wild type) | | 15 |
| Tyrosinase | HLA-A2 | MLLAVLYCL | 1-9 | 16 |
| | HLA-A2 | YMNGTMSQV | 369-377 | 17 |
| | HLA-A2 | YMDGTMSQV | 369-377 | 18 |
| | HLA-A24 | AFLPWHRLF | 206-214 | 19 |
| | HLA-B44 | SEIWRDIDF | 192-200 | 20 |
| | HLA-B44 | YEIWRDIDF | 192-200 | 21 |
| | HLA-DR4 | QNILLSNAPLGPQFP | 56-70 | 22 |
| | HLA-DR4 | DYSYLQDSDPDSFQD | 448-462 | 23 |
| Melan-A$^{MART-1}$ | HLA-A2 | (E)AAGIGILTV | 26/27-35 | 24 |
| | HLA-A2 | ILTVILGVL | 32-40 | 25 |
| gp100$^{Pmel117}$ | HLA-A2 | KTWGQYWQV | 154-162 | 26 |
| | HLA-A2 | ITDQVPFSV | 209-217 | 27 |
| | HLA-A2 | YLEPGPVTA | 280-288 | 28 |
| | HLA-A2 | LLDGTATLRL | 457-466 | 29 |
| | HLA-A2 | VLYRYGSFSV | 476-485 | 30 |
| DAGE | HLA-A24 | LYVDSLFFL | 301-309 | 31 |
| MAGE-A6 | HLA-Cw16 | KISGGPRISYPL | 292-303 | 32 |

As described herein above, sequences coding for
a full-length naturally occurring protein, a part of a
naturally occurring protein, combinations of parts of a
naturally occurring protein, or combinations of different
naturally occurring proteins or parts from different

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proteins, may all be employed to be cloned into the expression vectors as described hereinabove.

The present invention further provides recombinant expression vectors which can be employed in the present methods, including recombinant yersinia expression vectors, e.g., pMS111-YopE$_{130}$-MAGE-A1 and PMS111-YopE$_{130}$-MAGE-A4; recombinant vaccinia vectors, e.g., WR-MAGE-A1 and WR-MAGE-A4; recombinant canarypox viral vectors, e.g., ALVAC-MAGE-A1; recombinant adenoviral vectors, e.g., adeno-MAGE-A4; and retroviral vectors, e.g., M1-CSM.

To carry out the methods of the present invention, a sample containing T-cell precursors is obtained from a subject, typically, a blood sample from a human subject. The subject can be a cancer patient or an individual without cancer. The sample may be treated to concentrate T-cell precursors prior to stimulation.

The sample is contacted with a first antigen presenting cell expressing a protein, along with any other materials that may be appropriate, such as lymphokines. Upon contact, specific T-cell precursors are activated and begin to proliferate.

Cells in the sample are subsequently tested by contacting the cells with a second antigen presenting cell expressing the protein. The sample can be first diluted and distributed into microwells such that individual cells can be separately tested. CTL Cells which are specific for the protein, or "responding CTLs", can be identified and selected by a variety of standard assays such as a $^{51}$Cr release assay, a IFN-γ secretion assay, or a TNF production assay.

In a preferred embodiment of the present invention, the CTL cells thus selected are subject to at

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least one additional cycle of stimulation and testing
steps.

According to the present invention, the antigen
presenting cells used at one step can differ from the
cells used in a subsequent step, either in cell type or
in the expression system from which the protein is
expressed.

For testing the specificity of CTL responses
after stimulation, antigen presenting cells of a type
that expresses high amounts of class I HLA molecules are
preferred, e.g., EBV-transformed B cells.

In a preferred embodiment of the present
invention, one of the expression systems used by the
antigen presenting cell at one step (either stimulation
or testing), is different from at least one of the other
expression systems used in another step.

More Preferably, the antigen presenting cells
used at a stimulation step employ an expression system
different from that used in the immediately following
testing step.

The present invention provides examples of
combinations of different antigen presenting cells which
can be used for isolating specific CTL clones. According
to the present invention, CD8⁺ T lymphocytes obtained from
an individual can be stimulated in microwells with
autologous monocyte-derived dendritic cells infected with
a recombinant ALVAC canarypoxvirus encoding a protein of
interest. After several times of stimulation, an aliquot
of each microculture can then be tested for specific
lysis of autologous EBV-B cells infected with a
recombinant Vaccinia encoding the protein of interest.
The positive microcultures can then be diluted and
stimulated again with autologous EBV-B cells infected

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with a recombinant *Yersinia* encoding the protein of interest. Specific clones can be detected and thus isolated by testing for specific lysis of autologous EBV-B cells infected with a recombinant Vaccinia encoding the protein of interest. Thus, the combination of antigen presenting cells used in the foregoing procedure can be characterized as dendritic-ALVAC/EBV-B-Vaccinia/EBV-B-Yersinia/EBV-B-Vaccinia. Additional preferred combinations of antigen presenting cells which can be used in the present methods include: dendritic-Adeno/EBV-B-Vaccinia/EBV-B-Yersinia/EBV-B-Vaccinia, dendritic-ALVAC/EBV-B-Vaccinia/T cell-retroviral /EBV-B-Vaccinia. The present invention is not limited to the above exemplified combinations.

In a further aspect of the invention, the present invention contemplates CTL clones isolated by using the methods of the present invention.

In another embodiment, the present invention contemplates methods for identifying antigenic peptide epitopes of a protein. According to such method, CTL clones that recognize certain antigenic epitopes of a protein are isolated using the present method of isolating CTL clones, as described hereinabove. Such clones can then be used to identify the specific antigenic peptides as well as the presenting HLA molecules, using a variety of well-known procedures, for example, procedures described in Examples 7-11.

According to the methods of the present invention, the identification of an antigenic peptide epitope of a protein is based on the capacity of the peptide/HLA complex, at the surface of an antigen presenting cell, to activate the specific CTLs. The antigenic peptide epitopes thus identified likely

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represent the epitopes that are well processed and
adequately expressed at the cell surface *in vivo*.  By
using such method of the present invention, antigenic
peptide epitopes from proteins of the MAGE family have
been identified; namely, MAGE-A1 peptide 230-238
(presented by HLA-Cw3 and recognized by clone LB1137
462/F3.2), MAGE-A1 peptide 258-266 (presented by HLA-B53
and recognized by clone LB1801 456/H7.11), MAGE-A1
peptide 62-70 (presented by HLA-Cw2 and recognized by
clone LB 1118 466/D3.31), MAGE-A1 peptide 222-231
(presented by HLA-A28 and recognized by clone LB1801
456/H8.33), MAGE-A1 peptide 289-297 (presented by HLA-B7
and recognized by clone LB1803 483/G8.4), a MAGE-A3
peptide 114-122 (presented by HLA-B40 and recognized by
clone LB1841 526/F7.1), and a MAGE-A4 peptide 230-239
(presented by HLA-A2 and recognized by clone LB1137
H4.13).  See **Table 2**.  Among these, MAGE-A1 peptide 230-
238 (SAYGEPRKL (SEQ ID NO: 2)) has been previously
identified, but was found therein to be presented by a
different HLA molecule, HLA-Cw16 (U.S. Patent No.
5,558,995).

**TABLE 2**

| GENE | POSITION | PEPTIDE | MHC | CTL | SEQ ID |
|------|----------|---------|-----|-----|--------|
| MAGE-A1 | 230-238 | SAYGEPRKL | HLA-Cw3 | LB1137 462/F3.2 | 2 |
| MAGE-A1 | 258-266 | DPARYEFLW | HLA-B53 | LB1801 456/H7.11 | 42 |
| MAGE-A4 | 230-239 | GVYDGREHTV | HLA-A2 | LB1137 H4.13 | 44 |
| MAGE-A1 | 62-70 | SAFPTTINF | HLA-Cw2 | LB1118 466/D3.31 | 47 |
| MAGE-A1 | 222-231 | EVYDGREHSA | HLA-A28 | LB1801 456/H8.33 | 48 |
| MAGE-A3 | 114-122 | AELVHFLLL | HLA-B40 | LB1841 526/F7.1 | 55 |
| MAGE-A1 | 289-297 | RVRFFFPSL | HLA-B7 | LB1803 483/G8.4 | 57 |

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        Accordingly, another embodiment of the present
invention provides isolated CTL clones that are specific
for peptide/HLA complexes SAYGEPRKL(SEQ ID NO:
2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53,
GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO:
47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28,
AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID
NO: 57)/HLA-B7, respectively.

        In a preferred embodiment, the present
invention provides isolated CTL clones LB1137 462/F3.2,
LB1801 456/H7.11, LB1118 466/D3.31, LB1801 456/H8.33,
LB1137 H4.13, LB1841 526/F7.1 and LB1803 483/G8.4.

        In another embodiment, the present invention is
directed to the newly isolated antigenic peptides,
namely, DPARYEFLW(SEQ ID NO: 42) (MAGE-A1 258-266),
GVYDGREHTV(SEQ ID NO: 44) (MAGE-A4 230-239),
SAFPTTINF(SEQ ID NO: 47) (MAGE-A1 62-70), EVYDGREHSA(SEQ
ID NO: 48) (MAGE-A1 222-231), AELVHFLLL (SEQ ID NO: 55)
(MAGE-A3 114-122) and RVRFFFPSL (SEQ ID NO: 57) (MAGE-A1
289-297). Nucleic acid sequences encoding these peptides
are also contemplated.

        Another embodiment of the present invention is
directed to the isolated peptide/HLA complexes of the
present invention. Specifically, the present invention
provides isolated complex of peptide SAYGEPRKL(SEQ ID NO:
2) and HLA-Cw3, complex of peptide DPARYEFLW(SEQ ID NO:
42) and HLA-B53, complex of peptide GVYDGREHTV(SEQ ID NO:
44) and HLA-A2, complex of peptide SAFPTTINF(SEQ ID NO:
47) and HLA-Cw2, complex of peptide EVYDGREHSA(SEQ ID NO:
48) and HLA-A28, complex of AELVHFLLL (SEQ ID NO: 55) and
HLA-B40, and complex of RVRFFFPSL (SEQ ID NO: 57) and
HLA-B7.

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Once the presenting HLA molecule for an antigenic peptide epitope has been ascertained, a complex of the peptide and the HLA molecule can be made by a variety of methods. For example, the HLA molecule can be produced and isolated by any appropriate recombinant expression system, e.g., an *E. coli*-based expression system. Peptides can be made by, e.g., chemical synthesis or recombinant expression. The peptides and the HLA molecules can then be mixed *in vitro* under conditions that favor the formation of the HLA/peptide complexes. Such conditions are well known in the art. See, e.g., Garboczi et al. (*Proc. Natl. Acad. Sci. USA* 89: 3429-3433, 1992 and Altman et al. (*Science* 274: 94-96, 1996).

The present invention further contemplates cells expressing any of the instant peptide/HLA complexes at the cell surface. Such cells can be made using any antigen presenting cells that are appropriate including cell lines (e.g., COS cells, CHO cells and the like), and by, e.g., peptide loading, or cotransfection as described in the Examples of the present disclosure.

In another embodiment, the present invention contemplates pharmaceutical compositions which include any one of the isolated CTL clones, the isolated antigenic peptides, the isolated peptide/HLA complexes, the antigen presenting cells expressing peptide/HLA complexes of the present invention, or combinations thereof.

The pharmaceutical compositions of the present invention can include other substances such as cytokines, adjuvants and pharmaceutically acceptable carriers. As used herein, a therapeutically acceptable carrier includes any and all solvents, including water,

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dispersion media, culture from cell media, isotonic agents and the like that are non-toxic to the host. Preferably, it is an aqueous isotonic buffered solution with a pH of around 7.0. The use of such media and agents in therapeutic compositions is well known in the art. Supplementary active ingredients can also be incorporated into the compositions.

In a further aspect of the present invention, the isolated CTL clones, the isolated antigenic peptides, the cells expressing the peptide/HLA complexes of the present invention are employed in various methods for diagnosing a pathological condition in a subject, preferably, a human subject.

The pathological conditions contemplated by the present invention include tumors and infections by pathogens such as bacteria, parasites, fungus or virus, and the like.

The term "abnormal expression" as used herein refers to an expression that is not present in normal cells or an expression that is present in normal cells at a significantly lower level. In the present invention, "an abnormal expression" can also be used to refer to an unusual processing of a protein which gives rise to an antigenic epitope that is not presented at the surface of normal cells.

In one embodiment, the present invention provides methods of diagnosing in a subject, a pathological condition characterized by an abnormal expression a peptide/HLA complex, by detecting in the subject, the presence of cells abnormally expressing such complex.

In another embodiment, the present invention provides methods of detecting in a subject the presence

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of cells abnormally expressing a peptide/HLA complex of the present invention, by using an isolated CTL clone specific for such complex.

According to the present invention, a sample containing the cells suspected to be abnormal is obtained from the subject by, e.g., tissue biopsy. The sample is then contacted with a CTL clone of the present invention. The presence of the abnormal cells can be determined by measuring the activity of the CTL clone (i.e., CTL response) using standard assays such as $^{51}$Cr release, IFN-gamma secretion, or TNF production.

In another embodiment, the present invention provides methods for detecting in a subject, the presence of CTL cells specific for an isolated peptide/HLA complex of the present invention. More specifically, a blood sample is secured from the subject and contacted with cells expressing the specific peptide/HLA complexes. The presence of CTL cells specific for the complex can be detected by any of the approaches described hereinabove, e.g., the lysis of the cells expressing the specific peptide/HLA complexes measurable by a standard $^{51}$Cr release assay.

Furthermore, the frequency of CTLs specific for a peptide/HLA complex can be assessed by, e.g., limiting dilution analysis or tetramer assays. By comparing with a normal individual, an increased frequency of CTLs specific for a peptide/HLA complex in an individual, is indicative of a pathological condition characterized by an abnormal expression of the complex. Accordingly, the present invention contemplates methods of diagnosing a pathological condition characterized by an abnormal expression of a peptide/HLA complex by detecting an

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increased frequency of CTLs specific for such peptide/HLA complex.

In a further aspect of the present invention, the isolated CTL clones, the isolated antigenic peptides, the cells expressing the peptide/HLA complexes of the present invention are employed in various methods for treating a pathological condition in a subject, preferably, a human subject.

The term "treating" is used to refer to alleviating or inhibiting a pathological condition, e.g., inhibiting tumor growth or metastasis, reducing the size of a tumor, or diminishing symptoms of a pathogen infection, by e.g., eliciting an immune response.

In one embodiment, an isolated CTL clone of the present invention can be administered, in a therapy regimen of adoptive transfer, to a subject suffering a pathological condition characterized by an abnormal expression of the peptide/HLA complex that is specifically recognized by such CTL clone.  See teachings by Greenberg (1986) *J. Immunol. 136 (5)*:  1917; Riddel et al. (1992) *Science 257*:  238; Lynch et al. (1991) *Eur. J. Immunol. 21*:  1403; and Kast et al. (1989) *Cell 59*: 603 for adoptive transfer.  CTLs, by lysing the cells abnormally expressing such antigens, can alleviate or treat the pathological condition at issue, such as a tumor and an infection with a parasite or a virus.

In another embodiment, the present invention provides methods of treating a subject suffering a pathological condition characterized by an abnormal expression of a peptide/HLA complex, by administering the isolated peptides, or the peptide/HLA complexes, to the subject.  The pathological condition can be alleviated

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by, e.g., specific immune responses elicited due to the administered peptides or peptide/HLA complexes.

In another embodiment of the present invention, a subject suffering a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention, can be treated by obtaining antigen presenting cells from the subject, modifying such cells to effect a presentation of the peptide/HLA complex at the cell surface, and then reperfusing such "loaded" cells into the subject. The modification can be achieved by transfecting the isolated antigen presenting cells with any appropriate expression vectors encoding the peptide or the full-length protein, or by loading the cells with the peptides at issue following a peptide loading procedure as described by, e.g., Nestle et al. (*Nature Medicine* 4: 328-332, 1998).

For treatment purposes, the isolated CTL clones, the peptides or the peptide/HLA complexes, or the cells expressing the peptide/HLA complexes, can be administered to a subject alone or in combination with other appropriate materials, such as cytokines, adjuvants or a pharmaceutical carriers. The amount of the CTL cells, the peptides, the peptide/HLA complexes, or cells expressing the complexes, can be determined according the condition of the subject.

For additional teachings of diagnostic and therapeutic uses of isolated CTLs and peptide/HLA complexes, see, e.g., Thomson et al. (1995) *PNAS 92*: 5845; Altman et al. (1996) *Science 274*: 94-96; Dunbar et al. (1998) *Current Biology 8*: 413-416; Greenberg et al. (1986) *J. Immunol. 136*: 1917; and Kast et al. (1989) *Cell 59*: 603-614.

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The present invention is further illustrated by the following examples.

All the publications mentioned in the present disclosure are incorporated herein by reference. The terms and expressions which have been employed in the present disclosure are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, recognizing that various modifications are possible within the scope of the invention.

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## EXAMPLE 1
### GENERATION OF RECOMBINANT *YERSINIA* AND TARGETING EBV-TRANSFORMED B CELLS WITH RECOMBINANT *YERSINIA*

**Strains, Plasmids and Growth Conditions**

      *Y.enterocolitica* strain E40(pYV40), MRS40(pYV40), which is the isogeneic ampicillin sensitive derivative of E40(pYV40), and their various non-polar mutants (Sory et al. (1995), *Proc. Natl Acad. Sci. USA 92*: 11998-12002). Plasmids are listed in **Table 3**. Bacteria were grown in Brain Heart Infusion (BHI) (Difco, Detroit, Michigan). After overnight preculture, bacteria were diluted 1/20 in fresh BHI, allowed to grow for 30 minutes at room temperature, and synthesis of the Yop virulon was induced by incubation for 150 minutes at 37°C before infection.

**Construction of the Polymutant *Yersinia* Strains**

      To construct the *yopHOPEM* polymutant strain, the *yopE*, *yopH*, *yopO*, *yopM* and *yopP* genes were successively knocked out by allelic exchange in the MRS40 strain using the suicide vectors pMRS101 and pKNG101. See, K. Kaniga et al. (1991) "A wide-host range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the blaA gene of *Yersinia enterocolitica*" *Gene 109*: 137-141 and M.R. Sarker et al. (1997) "An improved version of suicide vector pKNG101 for gene replacement in Gram-negative bacteria" *Mol. Microbiol. 23*: 409-411. The various deletions are described in Table 2 in the "suicide vectors and mutators" section. The *YopE* gene was first mutated using the mutator pPW52 (see, P. Wattiau et al. (1993) "SycE, a chaperone-like protein of *Yersinia enterocolitica*

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involved in the secretion of YopE" *Mol. Microbiol. 8*:
123-131), giving strain MRS40(pAB4052).  Mutation of the
*YopH* gene in this strain with the mutator pAB31 (see,
S.D. Mills et al. (1997) "*Yersinia enterocolitica* induces
apoptosis in macrophages by a process requiring
functional type III secretion and translocation
mechanisms and involving YopP, presumably acting as an
effector protein" *Proc. Natl. Acad. Sci. USA 94*:  12638-
12643) gave the double *yopEH* mutant MRS40(pAB404).  The
triple *yopEHO* mutant MRS40(pAB405) was then obtained by
allelic exchange with the mutator pAB34 (see, S.D. Mills
et al., 1997).  The *YopP* gene was then mutated with
mutator pMSK7 (see S.D. Mills et al. (1997)), leading to
the *yopEHOP* mutant MRS40(pMSK46).  The *yopHOPEM* strain
MRS40(pABL403) was finally obtained by allelic exchange
with the *yopM* mutator pAB38 (see, S.D. Mills et al.,
1997).


**Table 3:  Plasmids**

| Plasmids | Relevant Characteristics | References |
|---|---|---|
| pABL403 | pYV40 $yopE_{21}$, $yopH\Delta^{1-352}$ $yopO\Delta^{65-558}$, $yopP_{23}$, $yopM_{23}$ | see Example 2 of the present specification |
|  | Suicide Vectors and mutators |  |
| pKNG101 | $^{ori}R6K$ $^{sac}BR+$ $^{on}TRK2$ $^{str}AB+$ | K. Kaniga et al. (1991) *Gene 109*:  137-141. |
| pMRS101 | $^{ori}R6K$ $^{sac}BR+$ $^{on}TRK2$ $^{str}AB+$ $^{ori}ColE1$ *bla* + | M.R. Sarker and G.R. Cornelis (1997) *Mol. Microbiol. 23*:  409-411. |

| pAB31 | pMRS101 yopH$_{\Delta 1-352}$+ | S.D. Mills et al. (1997) *Proc. Natl. Acad. Sci. USA 94*: 12638-12643. |
|-------|--------------------------------|------------------------------------------------------------------------|
| pAB34 | pMRS101 yopO$_{\Delta 65-558}$+ | S.D. Mills et al. (1997) |
| pAB38 | pMRS101 yopM$_{23}$+ | S.D. Mills et al. (1997) |
| pMSK7 | pMRS101 yopP$_{23}$+ | S.D. Mills et al. (1997) |
| pPW52 | pKNG101 yopE$_{21}$+ | P. Wattiau and G.R. Cornelis (1993) *Mol. Microbiol. 8*: 123-131. |

## Generation of recombinant *Yersinia* containing YopE$_{130}$-MAGE-A1

The sequence encoding protein MAGE-A1 was inserted in frame with a sequence encoding a truncated YopE, YopE$_{130}$, containing the first 130 amino acids of YopE. Such a plasmid is graphically depicted in **Figure 1**.

The open reading frame of MAGE-A1 was amplified by PCR using a MAGE-A1 cDNA cloned in pcDNAI/Amp (Invitrogen, Carlsbad, California) as template. The upstream primer, AAACTGCAGATGTCTCTTGAGCAGAGGAGTC (SEQ ID NO: 33), consisted of the first nucleotides of the open reading frame of MAGE-A1 preceded by a PstI site. The downstream primer, AAACTGCAGTCAGACTCCCTCTTCCTCCTC (SEQ ID NO: 34), consisted of nucleotides complementary to the last nucleotides of the open reading frame of MAGE-A1 followed by a PstI site. The PCR product was digested with PstI and inserted in frame with the truncated *YopE* at the PstI site of vector pMS111 (see, Sory et al. (1994) *Molecular Microbiology 14*: 583-594), to yield plasmid YopE$_{130}$-MAGE-A1 or pMS111-MAGE-A1.

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pMS111-MAGE-A1 (YopE$_{130}$-MAGE-A1) was
electroporated in bacteria strain DH5 F'IQ.  DNA was
extracted from some clones and the DNA of a positive
recombinant clone was electroporated in bacteria strain
SM10.  After mobilization of pMS111 from SM10 in *Yersinia*
MRS40 (pABL403), recombinant clones were then selected on
agar-containing medium, supplemented with nalidixic acid,
sodium-arsenite and chloramphenicol.  MRS40 is an
isogeneic derivative of E40 sensitive to ampicillin (see,
Sory et al. (1995) *Proc. Natl. Acad. Sci. USA 92*:  11998-
12002).


**Generation of recombinant *Yersinia* containing YopE$_{130}$-**
**MAGE-A4**

           The sequence encoding protein MAGE-A4 was
linked in frame to a sequence encoding a truncated YopE,
YopE (1-130), containing the first 130 amino acids of
YopE.  The open reading frame of MAGE-A4 was amplified by
PCR using a MAGE-A4 cDNA cloned in pcDNAI/Amp
(Invitrogen) as template.  The upstream primer,
AAAAACTGCAGATGTCTTCTGAGCAGAAGAGT (SEQ ID NO: 35),
consisted of the first nucleotides of the open reading
frame of MAGE-A4 preceded by a PstI site.  The downstream
primer, AAAAAATCGATTCAGACTCCCTCTTCCTC (SEQ ID NO: 36),
consisted of nucleotides complementary to the last
nucleotides of the open reading frame of MAGE-A4 followed
by a ClaI site.  The PCR was performed for 30 cycles (1
min at 94°C, 2 min at 55°C and 2 min at 72°C).  The PCR
product was digested with PstI and ClaI and inserted in
frame with the truncated YopE at the PstI-ClaI sites of
vector pMS621.  Plasmid pMS621-MAGE-A4 was transformed
into bacteria strain DH5αF'IQ by electroporation.
Positive clones were detected by PCR on bacterial

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colonies and the DNA of a positive recombinant clone was
extracted and transformed into bacteria strain SM10 by
electroporation.  After mobilization of pMS621-MAGE-A4
from SM10 into polymutant *Yersinia* MRS40 (pABL403),
5  recombinant clones were then selected on agar-containing
medium, supplemented with nalidixic acid, sodium m-
arsenite and chloramphenicol.


**Generation of recombinant *Yersinia*-MAGE-A3**

10  Yersinia-MAGE-A3(aa147-314)
        The sequences encoding the truncated MAGE-A3
was amplified by PCR using a MAGE-A3 cDNA cloned in
pcDNAI/Amp as template.  The upstream primer PVB157 was
5'-AA **CTGCAG** TTTCCTGTGATCTTCAGCAAAGC-3'(SEQ ID NO: 50),
15  consisting of nucleotides 439-461 of the open reading
frame of MAGE-A3 (start codon ATG is 1-3), preceded by a
PstI site (in bold) and 2 A's.  The downstream primer
PVB139, 5'-CC **ATCGAT** TCACTCTTCCCCCTCTCTCAA-3'(SEQ ID NO:
51), consisting of nucleotides complementary to the last
20  nucleotides of the open reading frame of MAGE-A3 preceded
by a ClaI site (in bold) and 2C's.  PCR was performed for
30 cycles (1 min at 94°C, 2 min at 62°C and 2 min at
72°C).  The PCR products were treated as described for
MAGE-A4 hereinabove.
25  Construction of Yersinia-MAGE-A3(aa1-199)
        To obtain the sequence encoding the first 199
amino-acids of the MAGE-A3 protein, the primers were
designed as follows: upstream primer PVB172,  5'-
ACCAGAGTCATC **CTGCAG** ATGCCTCTTGAG -3' (SEQ ID NO: 52),
30  consisting of the nucleotides overlapping the start codon
(underlined) of the open reading frame of MAGE-A3
preceded by a PstI site (in bold).  The downstream primer

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PVB 173, 5'-GCCTGCCTTGGG **ATCGAT** TCACATGATCTGATT-3'(SEQ
ID NO: 53), consisting of nucleotides complementary to
the nucleotides 577-600 of the open reading frame of
MAGE-A3 and containing a ClaI site (in bold). PCR was
performed for 30 cycles (1 min at 94°C, 2 min at 65°C and
2 min at 72°C). The PCR products were treated as
described hereinabove for MAGE-A4.

**Targeting EBV-Transformed B Cells with Recombinant
*Yersinia***

Infection of EBV-transformed B cells with
*Yersinia* MRS40 (pABL403) containing pMS111-MAGE-A1 was
carried out as follows. Infection of EBV-transformed B
cells with other recombinant *Yersinia* was carried out
following essentiallly the same procedure.

One colony of *Yersinia* MRS40 (pABL403)
containing pMS111-MAGE-A1 was then grown overnight at
28°C in LB medium supplemented with nalidixic acid (35
µg/ml), sodium m-arsenite (1 mM) and chloramphenicol (12
µg/ml). The overnight culture was diluted in fresh
medium in order to obtain an OD (optical density) of 0.2
at 600nm after amplifying the fresh culture at 28°C for
approximately 2 hours. The bacteria were washed in 0.9%
NaCl and resuspended at $10^8$ bacteria per ml in 0.9% NaCl
assuming that a culture giving an $OD_{600}$ equal to 1
contains $5 \times 10^8$ bacteria per ml. Irradiated EBV-B cells
(100Gy) were resuspended at $10^6$ in 3.8 ml of RPMI without
antibiotics, supplemented with 10% FCS and AAG (L-
Arginine (116 mg/ml), L-Asparagine (36 mg/ml) and L-
Glutamine (216 mg/ml)). Then 200 µl of the bacterial
suspension was added. Two hours after infection,
gentamicin (30 $\mu$g/ml) was added for the next two hours,

and the cells were finally washed three times before being used as stimulator cells.

As a negative control, the same cells were also infected with *Yersinia* MRS40 (pABL403) containing pMS621, a plasmid which encodes only the truncated YopE, i.e., $YopE_{130}$.

EBV-B Cells infected with the recombinant *Yersinia*-MAGE-A1 were recognized by MZ2-CTL 82/30. MZ2-CTL 82/30 are specific for the MAGE-A1 peptide EADPTGHSY (SEQ ID NO: 1) which is presented by HLA-A1 (U.S. Patent No. 5,342,774). 5000 MZ2-CTL 82/30 cells were added in each microwell containing the *Yersinia* in a final volume of 100 $\mu$l of Iscove's complete medium (culture medium was supplemented with 10% human serum, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), streptomycine (0.1 mg/ml), penicillin (200 U/ml), IL-2 (25 U/ml) and gentamicin (15 $\mu$g/ml). After overnight incubation, the presence of IFN-gamma (that is produced by CTL upon activation) in the supernatant of the co-culture was tested in a standard ELISA assay (Biosource, Fleurus, Belgium). **Figure 2A** graphically depicts such a procedure.

As indicated in **Figure 2B**, the HLA-A1^{+} B cells infected with *Yersinia* encoding $YopE_{130}$-MAGE-A1 were recognized by the CTL 82/30, while the same cells infected with the control plasmid $YopE_{130}$ were not. The optimal concentration of bacteria is around 1,000,000 per microwell.

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## EXAMPLE 2
## GENERATION OF RECOMBINANT VACCINIA WR VIRUSES

Parental WR strain of Vaccinia (vP1170) contained the parent vector pKILGPT of 2826 bp (Virogenetics, Troy, New York). A sequence coding for MAGE-A1, placed after the Vaccinia Virus H6 promoter, was cloned into the pKILGPT vector, creating donor plasmid MAW035. A similar MAGE-A4 donor plasmid vector was constructed by replacing the MAGE-A1 cDNA with the MAGE-A4 cDNA. The MAGE-A3 cDNA was digested with Pst1 and XbaI, blunt ended, the insert was gel purified and ligated into the SmaI site of the PsC11 vector. For the PsC11 vector, see Chakrabati et al. (1985) *Mol. Cell Biol.* 5: 34-3-3409.

The donor plasmids was transfected into CEF cells containing the genomic DNA of vaccinia strain WR, yielding recombinant vaccinia viruses WR-MAGE-A1, WR-MAGE-A4 and WR-MAGE-A3, respectively, by way of *in vivo* recombination and selected with BrdU and X-gal. The procedure can be found in, e.g., Perkins et al. (1989) *J. Virol.* 63: 3829-3936.

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## EXAMPLE 3
## GENERATION OF RECOMBINANT ALVAC-MAGE-A1 VIRUSES

A MAGE-A1 coding sequence, placed after the
Vaccinia Virus H6 promoter, was cloned into the pUC8-
based vector to generate donor plasmid MAW036.

Recombinant ALVAC-MAGE-A1 virus was generated
by using the donor plasmid MAW036 and following well
known procedures, e.g., as described in *Current
Protocols in Molecular Cloning* (Ausubel et al., John
Wiley & Sons, New York) and Ferrari et al. (*Blood* 90:
2406-2416, 1997).

Recombinant canarypox virus ALVAC-MAGE-A3 split
(also referred to herein as "ALVAC-MAGE-A3" for
simplicity) expressed two truncated overlapping fragments
of MAGE-A3, one fragment spanning amino acids 1-196 and
the other fragment spanning amino acids 147-199. In the
recombinant virus, each fragment was contained in a
separate expression cassette, each under the control of
the vaccinia virus H6 promoter, and both cassettes were
inserted at the C3 site in the ALVAC genome.

The vCP1563 recombinant was generated as
follows. The MAGE-A3 (1-196) DNA fragment was generated
and linked to the vaccinia H6 promoter by standard PCR
procedures with plasmid pTZ18R (containing full length
MAGE-A3 cDNA) as template. The MAGE-A3 (147-199) DNA
fragment was generated and linked to the H6 promoter in
the same way. These two fragments were then subcloned
into a plasmid such that the cassettes were flanked by
ALVAC DNA from the C3 insertion site. The organization
of these elements in the plasmid was as follows: ALVAC C3
left flanking arm, MAGE-A3 (147-299)/H6, MAGE-A3 (1-
196)/H6, ALVAC C3 right flanking arm. This ALVAC C3 site
donor plasmid containing the MAGE-A3 (1-196) and (147-

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299) fragment expression cassettes was designated pC3MAGE3 split.

The ALVAC-MAGE-A3 recombinant was generated by *in vivo* recombination between the pC3MAGE3 split donor plasmid and ALVAC genomic DNA following standard procedures. Recombinant virus was selected by hybridization with MAGE-3-specific DNA probes and plaque was purified. The resulting ALVAC-MAGE-A3 recombinant was given the laboratory designation vCP1563. Expression analysis with MAGE-3-specific antisera confirmed the expression of MAGE-A3 (1-196) and (147-299) polypeptides in cells infected with ALVAC-MAGE-A3 (vCP1563).

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## EXAMPLE 4
## GENERATION OF RECOMBINANT ADENOVIRUSES

For the construction of the recombinant adenovirus, the plasmid pAd-CMVIcpA-MAGE-A4 (containing the MAGE-A4 cDNA under the control of the CMV promoter) was obtained by inserting into the NotI site of vector pAd-CMVIcpA (provided by Celia GARCIA and Thierry RAGOT, URA CNRS 1301), the MAGE-A4 complete cDNA.

The recombinant adenovirus Ad-MAGE-A4 was generated by *in vivo* homologous recombination in cell line 293 between pAd-CMVIcpA-MAGE-A4 and Ad-ßgal genomic DNA. Briefly, 293 cells were cotransfected with 5 $\mu$g of plasmid pAd-CMVIcpA-MAGE-A4 linearized with XmnI and 5 $\mu$g of the large ClaI fragment of Adeno-ßgal DNA (Stratford-Perricaudet et al. (1992), *J. Clin. Invest.*, 90: 626-630 and Patent FR 9603207. The recombinant adenovirus was plaque purified and the presence of the transgene was assessed by restriction analysis of the adenoviral DNA. Recombinant adenoviruses were propagated in 293 cells and purified by double cesium chloride density centrifugation. The viral stocks were stored in aliquots with 10% glycerol in liquid nitrogen and titered by plaque assay using 293 cells.

Recombinant adenovirus Ad-MAGE-A3 was generated according to essentially the same procedure described above, but MAGE-A3 cDNA was derived from a λgt10 recombinant clone.

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## EXAMPLE 5
## RECOMBINANT RETROVIRUS
## AND INFECTION OF CELL LINES

The M1-CSM retroviral vector encodes the full length MAGE-A1 protein, under the control of the LTR, and the truncated form of the human low affinity nerve growth factor receptor (ΔLNGFr) driven by the SV40 promoter (Mavilio F. et al., *Blood* 83: 1988-1997, 1994). EBV-B cells or PHA-activated T cells were transduced by coculture with irradiated packaging cell lines producing the M1-CSM vector in the presence of polybrene (8 µg/ml). After 72 hours, lymphocytes were harvested and seeded in fresh medium. The percentage of infected cells was evaluated 48 hours later by flow cytomemtry for LNGFr expression with the mAb 20.4 (ATCC, Rockville, MD, USA). The LNGFr positive cells were purified by magnetic cell sorting using Rat anti-mouse IgG1-coated beads (Dynabeads M-450, DYNAL A.S. N012 Oslo, Norway).

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**EXAMPLE 6**
**MATERIALS AND METHODS**

**Cell lines and Media**

        The Epstein Barr Virus (EBV) immortalized B
cells (hereafter referred as to EBV-B cells) were
obtained following the standard protocol.  EBV-B cells
and the melanoma cell lines were cultured in Iscove's
modified Dulbecco medium (IMDM) (GIBCO BRL, Baitherburg,
MD, USA) supplemented with 10% fetal calf serum (FCS)
(GIBCO BRL), 0.24 mM L-asparagine, 0.55 mM L-arginine,
1.5 mM L-glutamine (AAG), 100 U/ml penicillin and 100
μg/ml streptomycin.  Hela and COS-7 cells were maintained
in H16 medium (GIBCO BRL) supplemented with 10% FCS.

**Cytokines**

        Human recombinant IL-2 was purchased from
CHIRON BV (Amsterdam, Netherlands) or EUROCETUS
(Amsterdam, Netherlands), or provided by BIOGEN (Geneva,
Switzerland).  Human recombinant IL-7 was purchased from
GENZYME (Cambridge, MA).  Human recombinant GM-CSF was
purchased from SANDOZ (Leucomax, Sandoz Pharma, Basel,
Switzerland) or SCHERING PLOUGH (Brinny, Ireland).  Human
recombinant IL-4, IL-6 and IL-12 were produced by the
present inventors.

**Processing of human blood**

        Peripheral blood was obtained from the local
blood bank (non cancer patients, namely, hemochromatosis
patients) as standard buffy coat preparations.
Peripheral blood mononuclear cells (PBMC) were isolated
by centrifugation on Lymphoprep (NYCOMED PHARMA, Oslo,
Norway).  In order to minimize contamination of PBMC by

platelets, the preparation was first centrifuged for 20
min at 1000 rpm at room temperature. After removal of
the top 20-25 ml containing most of the platelets, the
tubes were centrifuged for 20 min at 1500 rpm at room
temperature. PBMC were depleted of T cells by rosetting
with sheep erythocytes (BIO MÉRIEUX, Marcy-1'Etoile,
France) treated with 2-aminoethyl-isothiouronium (SIGMA,
St. Louis, MO, USA). Rosetted T cells were treated with
NH$_4$Cl (160 mM) to lyse the sheep erythrocytes and washed.
The CD8$^+$ T lymphocytes were isolated by positive selection
using an anti-CD8 monoclonal antibody coupled to magnetic
microbeads (MILTENYI BIOTECH, Germany) and by sorting
through a magnet. The CD8$^+$ T lymphocytes were frozen, and
thawed the day before the start of the primary culture
and cultured overnight in Iscove's medium containing L-
asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine
(1.5 mM), 10% human serum (hereafter referred to as
complete Iscove's medium) and supplemented with 2.5 U/ml
IL-2.

The lymphocyte-depleted PBMC were frozen or
used immediately for dendritic cell cultures. Cells were
left to adhere for 1-2 hrs at 37°C in culture flasks
(Falcon, BECTON DICKINSON LABWARE, Franklin Lakes, USA)
at a density of 2 x 10$^6$ cells/ml in RPMI 1640 medium
(GIBCO BRL) supplemented with L-asparagine (0.24 mM), L-
arginine (0.55 mM), L-glutamine (1.5 mM) and 10% fetal
calf serum (hereinafter referred to as complete RPMI
medium). Non-adherent cells were discarded and adherent
cells were cultured in the presence of IL-4 (100 U/ml)
and GM-CSF (100 ng/ml) in complete RPMI medium. For
experiments in Examples 7 and 8, cultures were fed on day
2 and day 4 by removing 1/3 of the volume of the medium
and adding fresh medium with IL-4 (100 U/ml) and GM-CSF

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(100 ng/ml); and on day 6 or 7, the non-adherent cell
population was used as a source of enriched dendritic
cells.  For experiments in Example 9, cultures were fed
on day 2 by removing 1/3 of the volume of the medium and
adding fresh medium with IL-4 (100 U/ml) and GM-CSF (100
ng/ml) and were frozen on day 4; and on the day before
each stimulation, dendritic cells were thawed and grown
overnight in complete medium supplemented with 100 U/ml
IL-4 and 100 ng/ml GM-CSF.  For experiments in Examples
10-11, cultures were fed on day 2 and 4 by removing 1/2
or 1/3 of the volume of the medium and adding fresh
medium with IL-4 (100 U/ml) and GM-CSF (100 ng/ml); and
on day 5 or day 7, the non-adherent cell population was
used as a source of enriched dendritic cells.


**Interferon γ production assay.**

        5000 target cells were cultured overnight with
2000 CTL in 100 $\mu$l per well complete Iscove's medium
supplemented with 25 U/ml IL-2 in 96 well round bottom
plates.  The production of interferon γ (IFN-γ) was
measured in 50 $\mu$l supernatant by ELISA (Biosource).


**cDNAs encoding HLA-class I molecules**

        The HLA-A*0201 coding sequence was obtained
from a cDNA library of cell line BB49, cloned into
expression vector pcDNAI/Amp (INVITROGEN).  The HLA-A3
coding sequence was isolated from a cDNA library of cell
line LB33 cloned into expression vector pcDNA3
(INVITROGEN).  The HLA-B*4402 coding sequence was
isolated by RT-PCR from cell line LB33 and cloned in
expression vector pcDNAI/Amp.  The HLA-B*40012 (B60)
coding sequence was derived by RT-PCR from cell line HA7-
RCC and cloned in expression vector pcDNA3.  The HLA-Cw3

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coding sequence was cloned in expression vector pCR3.
The HLA-Cw5 was isolated from cell line LB373 by RT-PCR
and cloned into pcDNA3. The HLA-B*0801, B*4002 (B61),
Cw*02022, and Cw*0701 coding sequences were amplified by
RT-PCR using RNA of LB 1118-EBV-B cells as the template.
The HLA-B*5301 coding sequence was amplified by RT-PCR
using RNA of EBV-B cells of patient LB 1118 as the
template. The HLA-B7 and HLA-B40 segments were isolated
as described in Examples 12 and 13. PCR products were
cloned into expression vector pcDNA3. DNA was extracted
from recombinant clones and sequenced partially on the
sense and partially on the antisense strand by the
dideoxy-chain termination method (Thermosequenase™ cycle
sequencing kit, Amersham).

**Peptides recognition assay**

        Peptides were synthesized on solid phase using
F-moc for transient NH2-terminal protection and were
characterized using mass spectrometry. All peptides were
>80% pure, as indicated by analytical HPLC. Lyophilized
peptides were dissolved in DMSO and stored at -20°C.
Target cells were labeled with $Na(^{51}Cr)O_4$, washed, and
incubated for 15 min in the presence of peptide. CTL
clone was then added at an effector-to-target ratio of
5:1 to 10:1. Chromium release was measured after
incubation at 37°C for 4 hours.

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## Example 7
## A MAGE-A1 DERIVED PEPTIDE PRESENTED BY HLA-Cw3
## MOLECULES TO CYTOLYTIC T LYMPHOCYTES

**Isolation of MAGE-A1 specific CTL clone LB1137 462/F3.2**

Autologous dendritic cells from donor LB 1137 (HLA-A2 A3 B4402 B60 Cw3 Cw5) were infected with the ALVAC-MAGE-A1 at a multiplicity of infection of 30 in RPMI containing 10% FCS at 37°C under 5% $CO_2$. After 2 hours, the infected dendritic cells were washed. For *in vitro* stimulation, 150,000 CD8^{+} T lymphocytes and 30,000 infected dendritic cells were cocultured in microwells in 200 $\mu$l Iscove's medium containing L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM), 10% human serum (hereafter referred to as complete Iscove's medium) and supplemented with IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The CD8^{+} lymphocytes were weekly restimulated with autologous dendritic cells freshly infected with the ALVAC-MAGE-A1 and grown in complete Iscove's medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml).

After several rounds of stimulation, an aliquot of each microculture was tested for specific lysis of autologous target cells. Autologous EBV-B cells were infected for two hours with either the parental vaccinia WR (batch LVAR) or the WR-MAGE-A1 construct (vP 1267), using a multiplicity of infection of 20, and labeled with Na($^{51}$Cr)$O_4$. Afterwards, EBV-B cells (target cells) were washed, and added to the responder cells at an effector to target ratio of approximately 40:1. Unlabeled K562 cells were also added (5 x 10$^4$ per V-bottomed microwell) to block natural killer activity. Chromium release was measured after incubation at 37°C for 4 hours. The individual microcultures were tested in duplicate on each target.

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The positive microcultures were cloned by limiting dilution, using autologous EBV-B cells infected with recombinant *Yersinia* expressing the $YopE_{130}$-MAGE-A1 protein as stimulating cells, and allogeneic EBV-B cells (LG2-EBV) as feeder cells. The cultures were restimulated similarly on day 7, and clones were maintained in culture by weekly restimulation with allogeneic EBV-B cells (LG2-EBV) in complete Iscove's medium supplemented with 0.5 μg/ml PHA-HA16 (Murex) and 50 U/ml of IL-2. At day 3 after restimulation, the clones were washed to remove the PHA-HA16 in the culture medium. The clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct. Clone LB1137 462/F3.2 was found positive (**Figure 3a**) and used in subsequent experiments.


**The MAGE-A1 epitope is presented to CTL by HLA-Cw3 molecules**

As donor LB 1137 expresses a number of different HLA molecules as described supra, each HLA was tested to determine which one presented the antigen recognized by CTL LB1137 462/F3.2.

COS cells were transfected with plasmids encoding one of the six HLA-class I molecules together with the cDNA of MAGE-A1. In brief, $1.5 \times 10^4$ COS cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNAI containing the MAGE-A1 cDNA and 50 ng of plasmid pcDNA3 containing the cDNA coding for one of the six HLA-class I molecules that were expressed by donor LB1137, using 1 μl of Lipofectamine reagent (Gibco BRL). The COS cells were incubated 5 hours at 37°C and 8% $CO_2$ in the transfection mixture and 200 μl of culture medium was added. After overnight culture, transfectants

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were tested for their ability to stimulate the production
of IFN-γ by clone LB1137 462/F3.2.  Briefly, 1500 CTLs
were added to each microwell containing transfected
cells, in a final volume of 100 µl of Iscove's complete
medium containing 25 U/ml of IL-2.  After 24 hours, 50 µl
supernatant was tested for its IFN-γ content in a WEHI
bioassay which measured the cytotoxic effect of IFN-γ on
cells of WEHI-164 clone 13 in a MTT colorimetric assay.
Only those cells transfected with both HLA-Cw3 and MAGE-
A1 stimulated CTL clone LB1137 462/F3.2 to produce IFN-γ
(**Figure 3b**).  COS cells transfected with MAGE-A1 or HLA-
Cw3 alone did not stimulate the CTL clone.

**Antigenic peptides and CTL assay.**

        In order to identify the MAGE-A1 peptide
recognized by clone LB1137 462/F3.2, peptides (16 amino-
acids) corresponding to parts of the MAGE-A1 protein were
synthesized, loaded on the autologous EBV-B cells and
tested for recognition.  Peptides were synthesized on
solid phase using F-moc for transient $NH_2$-terminal
protection.  Lyophilized peptides were dissolved at 20
mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid
and stored at -20°C.
        Peptides were tested in chromium release assays
in which 1000 $^{51}$Cr-labeled target cells were incubated
with 10 $\mu$g/ml of peptide in 96-well microplates (100
µl/well) for 20 min at room temperature, prior to adding
100 µl medium containing 10,000 CTL.  The assay was
terminated after 4 hours of incubation at 37°C and 8%
CO2.
        Autologous EBV-B cells incubated with peptide
DGREHSAYGEPRKLLT (MAGE-A1 $_{225-240}$) (SEQ ID NO: 37) were
recognized by CTL LB1137 462/F3.2 (Figure 3c).  This long

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peptide contained a 9-amino-acid peptide SAYGEPRKL (MAGE-A1 $_{230-238}$) (SEQ ID NO: 2) which contained adequate anchor residues for HLA-Cw3: a Y in position 3 and a L at the C-terminus. DGREHSAYGEPRKLLT (SEQ ID NO: 37) was screened

5      for prediction of an HLA-Cw3 binding peptide with the software available at "http://bimas.dcrt.nih.gov/molbio/hla_bind /index.html". Peptide SAYGEPRKL (MAGE-A1 $_{230-238}$) (SEQ ID NO: 2) had the highest score for binding to HLA-Cw3. It was recognized

10     by CTLLB1137 462/F3.2 in a cytotoxicity assay at an effector to target ratio of 10:1 (**Figure 3c**).


**Recognition by CTL clone LB1137 462/F3.2 of HLA-Cw3 positive tumor cells expressing MAGE-A1**

15           The activation of CTL LB1137 462/F3.2 by tumor cell lines that express HLA-Cw3 and MAGE-A1 was tested in an IFN-γ production assay. CTL clone LB1137 462/F3.2 recognized the HLA-Cw3 positive tumor cell line LB17-MEL which expresses MAGE-A1 (**Figure 3d**). The melanoma cell

20     line Mi 665/2 E+ clone 2, that was transfected with a genomic fragment containing the open reading frame of MAGE-A1 (as described in U.S. Patent NO. 5,342,774), was also recognized by clone LB1137 462/F3.2, whereas the parental cell line Mi 665/2 was not recognized.

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## Example 8
## A MAGE-A1 DERIVED PEPTIDE PRESENTED BY HLA-B5301
## MOLECULES TO CYTOLYTIC T LYMPHOCYTES

**Isolation of MAGE-A1 specific CTL clone LB1801 456/H7.11**

Autologous dendritic cells from donor LB1801
(HLA-A201, A28, B4401, B5301, Cw04, Cw0501) were
infected with the ALVAC-MAGE-A1 construct at a
multiplicity of infection of 30 in RPMI containing 10%
FCS at 37°C under 5% $CO_2$. After 2 hours, the infected
dendritic cells were washed twice. For *in vitro*
stimulation, 150,000 CD8+ lymphocytes and 30,000 infected
dendritic cells were cocultured in round bottomed
microwells in 200 microliters Iscove's medium containing
L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine
(1.5 mM), 10% human serum (hereafter referred as complete
Iscove's medium) and supplemented with IL-6 (1000 U/ml)
and IL12 (10 ng/ml). The CD8+ lymphocytes were weekly
restimulated with autologous dendritic cells freshly
infected with the ALVAC-MAGE-A1 construct and grown in
complete Iscove's medium supplemented with IL-2 (10 U/ml)
and IL-7 (5 ng/ml).

Autologous EBV-B cells were infected for 2
hours with either the parental vaccinia WR (vP1170) or
the recombinant vaccinia WR-MAGE-A1 (vP1188) using a
multiplicity of infection of 20, and labeled with
Na($^{51}$Cr)$O_4$. Target cells were washed, and added to the
responder cells at an effector to target ratio of
approximately 40:1. Unlabeled K562 were also added (5 x
$10^4$ per V-bottomed microwell) to block natural killer
activity. Chromium release was measured after incubation
at 37°C for 4 hours. The individual microcultures were
tested in duplicate on each target.

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The microcultures containing cells that specifically lysed autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct were cloned by limiting dilution using autologous EBV-B cells previously infected with the *Yersinia* expressing $YopE_{1-130}$-MAGE-A1 as stimulating cells, and allogeneic EBV-B cells (LG2-EBV) as feeder cells. CTL clones were maintained in culture by weekly restimulation in complete Iscove's medium supplemented with 50 U/ml of IL2. The clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct. Clone LB1801 456/H7.11 was found positive (Figure 4a) and used in the following experiments. The CTL was restimulated weekly with LG2-EBV as feeder cells and alternately, purified phytohaemagglutin (PHA-HA16; MUREX) (0.5 mg/ml) or autologous EBV-B cells previously infected with the *Yersinia*-$YopE_{1-130}$-MAGE-A1.

**Antigenic peptides and CTL assay**

In order to identify the MAGE-A1 peptide recognized by clone LB1801 456/H7.11, peptides (16 amino-acids) corresponding to parts of the MAGE-A1 protein were synthesized, loaded on the autologous EBV-B cells and tested for recognition. Peptides were synthesized on solid phase using F-moc for transient NH2-terminal protection. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid and stored at -20°C. Peptides were tested in chromium release assay where 1000 $^{51}Cr$-labeled target cells were incubated for 15 min at room temperature in V-bottomed microplates with 5 $\mu$g/ml of peptide, before adding an equal volume containing 5,000 CTLs. The assay was terminated after 4 hours of incubation at 37°C and 8%

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CO2. Peptides QVPDSDPARYEFLWGP (MAGE-A1 253-268) (SEQ ID
NO: 38) and SDPARYEFLWGPRALA (MAGE-A1 257-272) (SEQ ID
NO: 39) scored positive.

**Identification of the HLA presenting molecule**

            To know which HLA molecule presented both 16-
mers peptides to CTL clone LB1801 456/H7.11, peptides
were tested in a chromium release assay using, as target
cells, EBV-B cells from different donors that shared HLA
molecules with donor LB1801. Clone LB1801 456/H7.11 were
able to recognize the peptide only when presented by
autologous cells (**Table 4**). Because, no EBV-B cells
expressing the HLA-B5301 molecule was tested, the cDNA
coding for HLA-B5301 of donor LB1801 was isolated.

            The HLA-B5301 coding sequence was amplified by
RT-PCR using RNA of LB1801-EBV-transformed B cells as
template. The PCR products were cloned into expression
vector pcDNA3 (Invitrogen BV, the Netherlands). DNA was
extracted from recombinant clones and sequenced partially
on the sense and partially on the antisense strand to
check that it was a sequence encoding HLA-B5301. The
sequence for HLA-B5301 is described by Mason and Pasham
(1998), *Tissue Antigens* 51: 417-466.

            COS-7 cells were transfected with plasmids
encoding HLA-B5301 molecule together with MAGE-A1 cDNA.
In brief, $1.5 \times 10^4$ COS-7 cells distributed in microwells
were cotransfected with 100 ng of plasmid pcDNAI
containing the MAGE-A1 cDNA, 100 ng of plasmid pcDNA3
containing the cDNA coding for HLA-B5301 molecule of
donor LB 1801, and one microliter of lipofectamine (Gibco
BRL). The COS-7 cells
were incubated 24 hours at 37°C and 8% $CO_2$. These
transfectants were then tested for their ability to

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stimulate the production of TNF by clone LB1801
456/H7.11.  Briefly, 1,500 CTLs were added to the
microwells containing

**Table 4**

| Target Cells | HLA Typing | | | | % of Lysis | |
|---|---|---|---|---|---|---|
| | | | | | No Peptide | SDPARYEFLWGPRALA |
| LB1801 | A2 A28 | B4402 B53 | CwD4 Cw0501 | | 5 | 41 |
| LB1118 | A2 A3 | B8 B61 | Cw2 Cw7 | | 19 | 15 |
| LB33 | A24 A28 | B13 B4402 | Cw6 Cw7 | | 22 | 18 |
| LB1158 | A2 A3 | B35 B51 | Cw1 Cw4 | | 6 | 4 |
| LB1137 | A2 A3 | B4402 B60 | Cw3 Cw5 | | 5 | 3 |
| LG2 | A24 A32 | B3503 B4403 | Cw4 | | 1 | 4 |
| LB1819 | A2 | B44 B57 | Cw5 Cw7 | | 0 | 4 |
| LB1161 | A3 A26 | B39 B4402 | | | 1 | 8 |
| LB1213 | A24 | B18 B35 | Cw4 Cw7 | | 0 | 4 |

Table 4:  Lysis by CTL LB1801 456/H7.11 of various EBV-B cells
(target cells) pulsed with MAGE-A1 peptide.
        EBV-B cells were 51Cr labeled and incubated with CTL at
an effector to target cell ratio of 5/1 in the presence (or not) of 5
microgrammes of peptide SDPARYEFLWGPRALA (SEQ ID NO: 39).  Chromium
release was measured after 4 hours.

the transfectants, in a total volume of 100 ml of
Iscove's complete medium containing 25 U/ml of IL-2.
After 24 hours,  the supernatant was collected and its
TNF content was determined by testing its cytotoxic
effect on cells of WEHI-164 clone 13 in a standard MTT

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colorimetric assay. The cells transfected with both HLA-B53 and MAGE-A1 stimulated CTL clone LB1801 456/H7.11 to produce TNF (**Figure 4b**). COS-7 cells transfected with MAGE-A1 or HLA-B53 alone did not stimulate the CTL clone.

**Identification of the antigenic peptide**

To identify the sequence of the shortest synthetic peptide recognized by clone LB1801 456/H7.11, we compared the lysis by the CTL of autologous EBV-B cells, loaded with the MAGE-A1 peptide SDPARYEFLWGPRALA (MAGE-A1 257-272) (SEQ ID NO: 39) or the MAGE-A4 peptide GSNPARYEFLWGPRAL (MAGE-A4 264-279) (SEQ ID NO: 40), in a chromium release at an effector target ratio of 10 and a final concentration of peptide of 5 µg/ml. The MAGE-A1 peptide, but not the MAGE-A4 peptide, was recognized. The 10-mer peptide SDPARYEFLW (SEQ ID NO: 41) and the 9-mer peptide DPARYEFLW (SEQ ID NO: 42) were then synthesized and tested in a cytotoxic assay at an effector to target ratio of 5. Both peptides were recognized. The shorter peptide was then tested at different concentration at an effector to target ratio of 10 (**Figure 4c**). Half-maximal lysis was obtained at between 10 and 100 ng/ml.

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## Example 9
## A MAGE-A4 DERIVED PEPTIDE PRESENTED BY HLA-A2
## MOLECULES TO CYTOLYTIC T LYMPHOCYTES

**Isolation of MAGE-A4 specific CTL clone LB1137 H4.13**

Autologous dendritic cells from donor LB 1137
(HLA-A2, -A3, -B4402, -B60, -Cw3, -Cw5) were infected
with the Ad-MAGE-A4 construct at a multiplicity of
infection of 200 in RPMI containing 10% FCS at 37°C under
5% $CO_2$. After 2 hours, the infected dendritic cells were
washed. For *in vitro* stimulation, 150,000 CD8+ T
lymphocytes and 30,000 infected dendritic cells were
cocultured in microwells in 200 µl Iscove's medium
containing L-asparagine (0.24 mM), L-arginine (0.55 mM),
L-glutamine (1.5 mM), 10% human serum (hereafter referred
to as complete Iscove's medium) and supplemented with IL-
6 (1000 U/ml) and IL-12 (10 ng/ml). The CD8+ lymphocytes
were weekly restimulated with autologous dendritic cells
freshly infected with the Ad-MAGE-A4 construct and grown
in complete Iscove's medium supplemented with IL-2 (10
U/ml) and IL-7 (5 ng/ml) These cells were tested as
responder cells in the following assay.

Autologous EBV-B cells were infected for 2
hours with either the parental vaccinia WR parent (batch
vP1170 or batch L VAR) or the recombinant vaccinia WR-
MAGE-A4 (batch vP1545) using a multiplicity of infection
of 20, and labeled with Na($^{51}$Cr)O$_4$. These target cells
were washed, and added to the responder cells at an
effector to target ratio of approximately 40:1.
Unlabeled K562 cells were also added (5 x 10$^4$ per V-
bottomed microwell) to block natural killer activity.
Chromium release was measured after incubation at 37°C
for 4 hours. The individual microcultures were tested in
duplicate on each target.

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The microcultures containing cells that specifically lysed autologous EBV-B cells infected with the vaccinia-MAGE-A4 construct were cloned by limiting dilution using, as stimulating cells, autologous EBV-B cells infected with the recombinant *Yersinia* expressing YopE$_{1-130}$-MAGE-A4 (described above), and using allogeneic EBV-B cells (LG2-EBV) as feeder cells. Infection of EBV-B cells with *Yersinia* YopE$_{1-130}$-MAGE-A4 was done as follows: one colony of Yersinia MRS40 (pABL403) containing pMS621-MAGE-A4 (YopE$_{1-130}$-MAGE-A4) was grown overnight at 28°C in LB medium supplemented with nalidixic acid, sodium m-arsenite and chloramphenicol. From this culture, a fresh culture at an OD (600 nm) of 0.2 was then amplified at 28°C for approximately 2 hours. The bacteria were then washed in 0.9 % NaCl and resuspended at 10$^8$ bacteria per ml in 0.9 % NaCl. Irradiated EBV-B cells were infected at a multiplicity of infection of 20 in complete RPMI 1640 (culture media was supplemented with 10% FCS, and with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml). Two hours after infection, gentamycin (30 μg/ml) was added for the next two hours, and the cells were finally washed 3 times. CTL clones were maintained in culture by weekly restimulation with either *Yersinia* YopE$_{1-130}$-MAGE-A4 infected EBV-B cells, HLA-A2 melanoma cell line QUAR (LB1751-MEL) that expressed MAGE-A4, or PHA (0.5 μg/ml) in complete Iscove's medium supplemented with 50 U/ml of IL-2. The clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A4 construct. Clone LB1137 H4.13 was found positive (**Figure 5a**) and used in the following experiments.

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**The MAGE-A4 epitope is presented to CTL by HLA-A2 molecules**

The lysis by CTL clone LB1137 H4.13 of EBV-B cells infected with the vaccinia-MAGE-A4 construct was inhibited by addition of an anti-HLA-A2 monoclonal antibody but not by addition of an anti-HLA-A3 or an anti-HLA-B,C monoclonal antibody. This indicated that the MAGE-A4 epitope was presented by HLA-A2 molecules.

COS cells were transfected with plasmids encoding the HLA-A2 molecule together with the cDNA of MAGE-A4. In brief, $1.5 \times 10^4$ COS cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNAI containing the MAGE-A4 cDNA, 50 ng of plasmid pcDNA1/Amp containing the genomic DNA coding for the HLA-A2 molecule and 1 μl of DMRIEC (Gibco BRL). The COS cells were incubated 24 hours at 37°C and 8% $CO_2$. These transfectants were then tested for their ability to stimulate the production of TNF by clone LB1137 H4.13. Briefly, 2000 CTL were added to the microwells containing the transfectants, in a total volume of 100 μl of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 in a standard MTT colorimetric assay. The cells transfected with both HLA-A2 and MAGE-A4 stimulated CTL clone LB1137 H4.13 to produce TNF (**Figure 5b**). COS cells transfected with MAGE-A4 or HLA-A2 alone did not stimulate the CTL clone.

**Determination of the antigenic peptide**

In order to identify the MAGE-A4 peptide recognized by clone LB1137 H4.13, PCR reactions were performed using the MAGE-A4 cDNA as template, an upstream

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primer (S) consisting of the first nucleotides of the
open reading frame of MAGE-A4 and 8 downstream primers
(AS1 to AS8) **(Figure 6)**, separated from each other by
approximately 100-120 bp in the open reading frame of
MAGE-A4.  The PCR was performed for 30 cycles (1 min at
94°C, 2 min at 63°C and 3 min at 72°C.  This led to the
amplification of 8 fragments of MAGE-A4 of different
lengths (MAGE-A4(1) to MAGE-A4(8)), the longer one (MAGE-
A4(1)) containing the entire open reading frame of MAGE-
A4.  PCR products were ligated into the pcDNA3.1/V5/His-
TOPO vector and the recombinant vectors were transformed
into *E. coli* cells (Topo TA cloning kit, Invitrogen).
Colonies were analyzed by PCR and DNA of positive clones
was extracted and used to transfect HeLa cells together
with a plasmid encoding the HLA-A2 molecule.  Briefly, 2
x $10^4$ HeLa cells distributed in microwells were
cotransfected with 50 ng of plasmid pcDNA3.1/V5/His-TOPO
containing the MAGE-4A fragment, 50 ng of plasmid
pcDNA1/Amp containing the genomic DNA coding for the HLA-
A2 molecule and 1 µl of Lipofectamine (Gibco BRL).  The
HeLa cells were incubated 24 hours at 37°C and 8% $CO_2$.
These transfectants were then tested for their ability to
stimulate the production of TNF by clone LB1137 H4.13 as
described above.  Transfection with inserts S-AS1 and S-
AS2 were positive, transfections with the other
constructs were negative. This led to the identification
of a MAGE-4A fragment of 130 bp,
TGATGGGAGGGAGCACACTGTCTATGGGGAGCCCAGGAAACTGCTCACCCAAGATTG
GGTGCAGGAAAACTACCTGGAGTACCGGCAGGTACCCGGCAGTAATCCTGCGCGCTA
TGAGTTCCTGTGGGGT (SEQ ID NO: 43),
encoding the epitope recognized by clone LB1137 H4.13.

          The sequence of the putative fragment of the
MAGE-A4 protein encoded by this region was screened for

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prediction of an HLA-A2 binding peptide with the software available at http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html". Peptide GVYDGREHTV(SEQ ID NO: 44) (MAGE-A4 $_{230-239}$) had the highest score. It was synthesized and tested in a cytotoxicity assay at an effector to target ratio of 10:1. Peptide GVYDGREHTV (MAGE-A4 $_{230-239}$)(SEQ ID NO: 44) was found to sensitize autologous target cells to lysis by clone LB1137 H4.13 (**Figure 5c**).

**Recognition by CTL clone LB1137 H4.13 of HLA-A2 cells expressing MAGE-A4**

As indicated in **Figure 5d**, CTL clone LB1137 H4.13 was able to lyse HLA-A2 melanoma cell line QUAR (LB1751-MEL) that expressed MAGE-A4.

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## EXAMPLE 10
## A MAGE-A1 PEPTIDE PRESENTED BY
## HLA-Cw2 TO CTL CLONE LB1118 466/D3.31

**Isolation Of CTL Clone LB1118 466/D3.31**

Dendritic cells ($3 \times 10^6$/ml) from donor LB 1118
(HLA-A*0201, A3, B*0801, B*4002, Cw*02022, Cw*0701) were
infected with the ALVAC-MAGE-A1 at a multiplicity of
infection of 30 in RPMI supplemented with AAG and 10% FCS
at 37°C under 5% $CO_2$. After 2 hours, the infected
dendritic cells were washed. 150,000 autologous CD8$^+$ T
lymphocytes and 30,000 infected dendritic cells were
cocultured in microwells in 200 $\mu$l complete Iscove's
medium and supplemented with IL-6 (1000 U/ml) and IL-12
(10 ng/ml). The CD8$^+$ lymphocytes were restimulated on
days 7 and 14 with autologous dendritic cells freshly
infected with the ALVAC-MAGE-A1 and grown in complete
Iscove's medium supplemented with IL-2 (10 U/ml) and IL-7
(5 ng/ml).

The microcultures containing proliferating CD8+
T cells were assessed on day 21 for their capacity to
lyse autologous EBV-B cells infected with vaccinia-MAGE-
A1 (vP 1188). EBV-B cells infected with parental
vaccinia (vP1170) were used as a negative control.
Infected EBV-B cells (target cells) were washed and added
to the responder cells at an effector to target ratio of
approximately 40:1. Unlabeled K562 cells were also added
($5 \times 10^4$ per V-bottomed microwell) to block natural killer
activity. Chromium release was measured after incubation
at 37°C for 4 hours. The individual microcultures were
tested in duplicate on each target. In a first
experiment, an anti-MAGE-A1 reactivity was detected in 3
microcultures out of 96. 13% of the microcultures
contained responder cells that lysed targets infected

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with either vaccinia or vaccinia-MAGE-A1, but not the
uninfected targets.  This result indicated that the ALVAC
and vaccinia vectors shared antigens recognized by CTL.
In a second experiment, 2 microcultures scored positive
in their anti-MAGE-A1 reactivity.

The positive microcultures (i.e., those that
recognize autologous EBV-B cells infected with vaccinia-
MAGE-A1 construct) were cloned by limiting dilution
using, as stimulating cells, either autologous PHA-
activated T cells transduced with a retrovirus encoding
MAGE-A1, or autologous EBV-B cells transduced with the
same retrovirus (5x10$^3$ to 10$^4$ cells per well in a 96-well
plate).  Allogeneic EBV-B cells (5x10$^3$ to 10$^4$ LG2-EBV-B
cells per well in a 96-well plate) were used as feeder
cells.  CTL clones were tested for specific lysis of
autologous EBV-B cells infected with the vaccinia-MAGE-A1
construct.  The established CTL clones were maintained in
complete IMDM supplemented with IL-2 (50 U/ml) and 0.5
µg/ml purified PHA (instead of stimulator cells) and
passaged by weekly restimulation with allogeneic EBV-B
cells (1.5x10$^6$ LG2-EBV-B cells per well in a 24-well
plate).

Clone LB1118 466/D3.31 was identified as a
positive clone that recognized autologous EBV-B cells
infected with vaccinia-MAGE-A1 (**Figure 7A**), or EBV-B
transduced with a retrovirus encoding MAGE-A1.

**Identification of the Peptide and the Presenting Molecule**

To identify the HLA molecule that presents the
MAGE-A1 peptide by CTL clone LB1118 466/D3.31, COS cells
were transfected with the MAGE-A1 cDNA, together with
cDNAs coding for one of the putative HLA presenting
molecules.  These transfected cells were then tested for

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their ability to stimulate the CTL clone to produce TNF.
CTL clone LB1118 466/D3.31 produced TNF upon stimulation
by COS cells transfected with MAGE-A1 and HLA-Cw2 (**Figure
7B**). To identify the MAGE-A1 peptide recognized by this

5       CTL clone, a set of MAGE-A1 peptides of 12 amino acids
that overlapped by 8 amino acids were screened.
Autologous EBV-B cells were incubated with each of these
peptides at a concentration of 1 µM, and tested for
recognition by the CTL in a chromium release assay.

10      Peptide ASAFPTTINFTR (MAGE-A1$_{61-72}$)(SEQ ID NO: 45) scored
positive whereas the 16 amino-acid peptide
SPQGASAFPTTINFTR (MAGE-A1$_{57-72}$), (SEQ ID NO: 46) scored
negative. As information was not available for the
residues anchoring a peptide in an HLA-Cw2 molecules, a

15      number of shorter peptides were tested. Peptide
SAFPTTINF (MAGE-A1$_{62-70}$) (SEQ ID NO: 47) was subsequently
found to be the shortest peptide capable of efficiently
sensitizing autologous target cells to lysis by CTL clone
LB1118 466/D3.31, with a half-maximal lysis obtained at

20      ~0.1nM (**Figure 7C**). The natural processing of the
antigen was shown by the lysis by CTL clone LB1118
466/D3.31 of HLA-Cw2 tumor cell lines that express MAGE-
A1 (**Figure 7D**).


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## EXAMPLE 11
## A MAGE-A1 PEPTIDE PRESENTED BY
## HLA-A28 TO CTL CLONE LB1801 456/H8.33

Dendritic cells were derived from donor LB 1801 (HLA-A*0201, A28, B*4401, B*5301, Cw4, Cw*0501). CTL clone LB1801 456/H8.33 was isolated by following essentially the same procedure as described in Example 10.

Briefly, immature dendritic cells derived from blood monocytes were infected with ALVAC-MAGE-A1 and used to stimulate autologous CD8+T cells in the presence of IL-6 and IL-12. Responder cells were restimulated once a week with autologous dendritic cells, infected with ALVAC-MAGE-A1, in the presence of IL-2 and IL-7. Responder cells were tested on day 28 for their lytic activity on autologous EBV-transformed B (EBV-B) cells infected with a vaccinia virus encoding MAGE-A1 (vaccinia-MAGE-A1).

Positive microcultures were subject to limiting dilution using EBV-B cells infected with *Yersinia*-MAGE-A1 as stimulating cells. CTL clone LB1801 456/H8.33 lysed autologous EBV-B cells infected with vaccinia-MAGE-A1 (**Figure 8A**). CTL clone LB1801 456/H8.33 produced TNF upon stimulation by COS-7 cells transfected with HLA-A28 and MAGE-A1 (**Figure 8B**). Peptide EVYDGREHSA (MAGE-A1$_{222-231}$)(SEQ ID NO: 48) produced half-maximal lysis of target cells at ~0.3 nM (**Figure 8C**). A tumor cell line expressing MAGE-A1 and HLA-A28 was lysed by the CTL, but the lysis was lower than that obtained with cells infected with vaccinia-MAGE-A1 (**Figure 8C**).

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## EXAMPLE 12
## A MAGE-A3 PEPTIDE PRESENTED BY HLA-B40 TO CTLS

**Processing of human blood**

Blood samples from donor LB 1841 (HLA-A3, -B35, -B40, Cw3, -Cw4) were processed as described in Example 6, except that:

1. the interphase containing the PBMC was harvested and then washed 3 times (or more) in cold phosphate buffer solution with 2 mM EDTA in order to eliminate the remaining platelets;

2. monocyte-derived dendritic cells (DC) were frozen on day 6. On the day of stimulation, DC were thawed and infected with a recombinant adenovirus encoding MAGE-A3, at a multiplicity of infection (MOI) of 500.

**Isolation of CTL clones specific for MAGE-A3**

Dendritic cells obtained from the blood sample of donor LB 1841 were infected with ALVAC-MAGE-A3 and cocultured with autologous CD8+ cells following essentially the same procedure as described in Example 7.

After several rounds of stimulation, the microculture was tested for specific lysis of autologous target cells following essentially the same procedure as described in Example 7, except that the vaccinia samples were sonicated for 30 seconds prior to use for infection of EBV-B target cells.

Microculture 526/F7, identified as containing cells that specifically lysed autologous EBV-B cells infected with vaccinia-MAGE-A3, was cloned by limiting dilution using, as stimulating cells, autologous EBV-B cells infected with recombinant ALVAC-MAGE-A3 (vCP1563)

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at a multiplicity of infection of 30 (the ALVAC-MAGE-A3
sample was also sonicated 30 sec before use), or with
PHA, according to the following scheme:

First week: stimulators = ALVAC-MAGE-A3
(vCP1563) infected EBV-B cells in complete IMDM medium
supplemented with 50 U IL-2/ml, 2.5 ng IL-12/ml and 5 U
IL-4/ml;

Second week: stimulators = ALVAC-MAGE-A3
(vCP1563) infected EBV-B cells in complete IMDM medium
supplemented with 50 U IL-2/ml;

Third week: PHA (0.5 µg/ml) in complete IMDM
medium supplemented with 50 U/ml of IL-2;

Fourth week: PHA (0.5 µg/ml) in complete IMDM
medium supplemented with 50 U/ml of IL-2, 5ng/ml of IL-7
+ gentamicin (15 µg/ml) + MRA 0.5 g/ml (from ICN);

Fifth and sixth week: stimulators = EBV-B cells
infected with Yersinia-MAGE-A3(aa147-314) and EBV-B cells
infected with Yersinia-MAGE-A3(aa1-199) in complete IMDM
medium supplemented with 50 U/ml of IL-2, 2.5ng/ml of IL-
12, 5 U/ml Il-4 + gentamicin (15 µg/ml);

Seventh week and each week thereafter: PHA (0.2
µg/ml) in complete IMDM medium supplemented with 50 U/ml
of IL-2, 5 U/ml of IL-4 + gentamicin (15 µg/ml).

CTL clones were maintained in culture by weekly
restimulation.

The clones were tested for specific lysis of
autologous EBV-immortalized B cells infected with the
vaccinia-MAGE-A3 construct, or with the parental vaccinia
as a negative control.  Clone LB1841 526/F7.1 was found
positive (**Figure 9A**) and was used in the following
experiments.

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**The MAGE-A3 epitope is presented to CTL LB1841 526/F7.1 by HLA-B40.**

To identify the HLA molecule that presents the MAGE-A3 epitope recognized by CTL clone LB1841 526/F7.1, COS cells were transfected with the MAGE-A3 cDNA together with cDNAs coding for each of the putative HLA presenting molecules. In brief, $1.5 \times 10^4$ COS cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNA3-MAGE-A3, 50 ng of plasmid containing coding sequences for HLA molecules and 1 µl of Lipofectamine (Gibco BRL). The HLA coding sequences were isolated from various individuals; in particular, the HLA-B40 cDNA was obtained by RT-PCR using RNA from tumor cell line HA-7-RCC as a template. This PCR product cDNA was inserted in pcDNA3 (InVitrogen). The COS cells were incubated 24 hours at 37°C and 8% $CO_2$. These transfectants were then tested for their ability to stimulate the production of TNF by clone LB1841 526/F7.1. Briefly, 2500 CTLs were added to the microwells containing the transfectants, in a total volume of 100 µl of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, the supernatant was collected and the TNF content was determined by testing the cytotoxic effect of the supernatant on cells of WEHI-164 clone 13 in a standard MTT colorimetric assay. The cells transfected with both HLA-B40 and MAGE-A3 stimulated CTL clone LB1841 526/F7.1 to produce TNF (**Figure 9B**). Cos cells transfected with MAGE-A3 or HLA-B40 alone did not stimulate the CTL clone.

**Identification of the antigenic peptide**

To identify the MAGE-A3 peptide recognized by CTL clone LB1841 526/F7.1, a set of peptides of 16 amino acids that overlapped by 12 amino acids and covered the

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entire MAGE-A3 protein sequence, was screened.
Autologous EBV-B cells were incubated with each of these
peptides at a concentration of 1 µg/ml, and tested for
recognition by CTL clone LB1841 526/F7.1 in a chromium
release assay at an effector to target cell ratio of 5:1.
Peptide AALSRKVAELVHFLLL (SEQ ID NO: 54) scored positive.
The sequence of this peptide was screened for prediction
of an HLA-B40 binding peptide with the software available
at
"http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html".
Peptide AELVHFLLL (MAGE-A3 114-122) (SEQ ID NO: 55) had
the highest score.  It was tested in a cytotoxicity assay
with CTL clone LB1841 526/F7.1 and produced half-maximal
lysis of autologous EBV-B target cells at ~7nM (**Figure
9C**).

**Recognition by CTL clone LB1841 526/F7.1 of HLA-B40 cells
expressing MAGE-A3**

CTL clone LB1841 526/F7.1 was also able to lyse
melanoma cell lines that expresses MAGE-A3 which were
obtained from HLA-B40 positive patients, e.g., LB-43-MEL
(**Figure 9D**).  Some melanoma cells, e.g., SK-MEL-28 and
SK-31-MEL, were not lysed by clone LB1841 526/F7.1, even
though the level of expression of MAGE-A3 appeared to be
appropriate in these cells.  The peptide-pulsed cells
were also insensitive to the CTL lysis.  The lack of
lysis was likely due to downregulation of the HLA
expression in these melanoma cells, since treatment of
SK-MEL-28 cells with IFN-γ increased the lysis of these
cells by clone LB1841 526/F7.1 (IFN-γ is known to
upregulate HLA expression in certain cells).

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## EXAMPLE 13
## A MAGE-A1 PEPTIDE PRESENTED BY HLA-B7
## MOLECULES TO CYTOLYTIC T LYMPHOCYTES

**Processing of human blood**

Blood samples from donor LB 1803 (HLA-A2, -A32, B7, -B60) were processed as described in Example 12.

**Isolation of CTL clones specific for MAGE-A1**

Dendritic cells obtained from the blood sample of donor LB 1803 were infected with ALVAC-MAGE-A1 and cocultured with autologous CD8+ cells following essentially the same procedure as described in Example 7 except that 15µg/ml gentamycin was added to the culture medium.

After several rounds of stimulation, the microculture was tested for specific lysis of autologous target cells following essentially the same procedure as described in Example 7, except that the vaccinia samples were sonicated for 30 seconds prior to use for infection of EBV-B target cells.

Microculture 483/G8, identified as containing cells that specifically lysed autologous EBV-B cells infected with recombinant vaccinia (WR-MAGE-A1), was cloned by limiting dilution using, for the first two weekly stimulations, irradiated autologous EBV-B cells infected with Yersinia-MAGE-A1 as stimulating cells, irradiated LG2-EBV as feeder cells, IL-2 (50 U/ml) and gentamycin (15 µg/ml). CTL clones were then maintained in culture by weekly restimulation with PHA (0.5 µg/ml), feeder cells and IL-2.

The clones were tested for specific lysis of autologous EBV-immortalized B cells infected with recombinant vaccinia (WR-MAGE-A1), or with the parental

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vaccinia as a negative control. Clone LB1803 483/G8.4
was found positive (**Figure 10A**) and was used in the
following experiments.

**The MAGE-A1 epitope is presented to CTL by HLA-B7
molecules.**

Donor LB 1803 was typed HLA-A2, -A32, -B7, -
B60. To identify the HLA molecule that presents the
MAGE-A1 epitope recognized by CTL clone LB1803 483/G8.4,
COS-7 cells were transfected with the MAGE-A1 cDNA
together with cDNAs coding for the putative HLA
presenting molecules. In brief, $1.5 \times 10^4$ COS-7 cells
distributed in microwells were cotransfected with 50 ng
of plasmid pcDNA3-MAGE-A1, 50 ng of plasmid containing
coding sequences for HLA molecules and 1 µl of
Lipofectamine (Gibco BRL). The HLA coding sequences were
isolated from various individuals; in particular, the
HLA-B7 cDNA was obtained by RT-PCR using RNA from tumor
cell line LB23-SAR as a template. This PCR product was
inserted in pcDSRalpha. The transfected COS-7 cells were
incubated 24 hours at 37°C and 8% $CO^2$. These
transfectants were then tested for their ability to
stimulate the production of TNF by clone LB1803 483/G8.4.
Briefly, 2000 CTL were added to the microwells containing
the transfectants, in a total volume of 100 µl of
Iscove's complete medium containing 25 U/ml of IL-2.
After 24 hours, the supernatant was collected and the TNF
content was determined by testing the cytotoxic effect of
the supernatant on cells of WEHI-164 clone 13 in a
standard MTT colorimetric assay. The cells transfected
with both HLA-B7 and MAGE-A1 stimulated CTL clone LB1803
483/G8.4 to produce TNF (**Figure 10B**). COS-7 cells
transfected with HLA-B7 and either MAGE-A2,-A3,-A4,-A6,-

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A8,-A9,-A10,-A11,-A12,-B1,-B2, or -C2 were unable to
stimulate CTL clone LB1803 483/G8.4 to produce TNF.


**Identification of the antigenic peptide**

5              To identify the MAGE-A1 peptide recognized by
CTL clone LB1803 483/G8.4, a set of peptides of 12 amino
acids, that overlapped by 8 amino acids and covered the
entire MAGE-A1 protein sequence, was screened.
Autologous EBV-B cells were incubated with each of these
10       peptides at a concentration of 2µg/ml, and tested for
recognition by CTL clone LB1803 483/G8.4 in a chromium
release assay at an effector to target cell ratio of
20:1. Peptide RVRFFFPSLREA (MAGE-A1 289-300) (SEQ ID NO:
56) scored positive. The sequence of this peptide was
15       screened for an HLA-B7 binding peptide with the software
available at
"http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html".
Peptide RVRFFFPSL (MAGE-A1 289-297) (SEQ ID NO: 57) had
the highest score. It was tested in a cytotoxicity assay
20       with CTL clone LB1803 483/G8.4 (E/T ratio of 20:1) and
produced half-maximal lysis of autologous EBV-B target
cells at ~ 20nM (**Figure 10D**).


**Recognition by CTL clone LB1803 483/G8.4 of HLA-B7 cells
25       expressing MAGE-A1.**

              CTL clone LB1803 483/G8.4 was also able to lyse
HLA-B7 melanoma cell line ME275 clone 2 that expressed
MAGE-A1 (**Figure 10D**). Melanoma cell line ME190DA was
also lysed but only after treatment for 72h with 100 U/ml
30       of IFN-gamma.

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**WE CLAIM:**

1.         A method for isolating, from a sample
containing T cells of a subject, a cytotoxic T cell clone
specific for a protein, comprising:

(a) contacting the sample with a first antigen
presenting cell, wherein the expression of said protein
in said first antigen presenting cell is effected by a
first expression system;

(b) testing cells in said sample with a second
antigen presenting cell and selecting for responding CTL
cells, wherein the expression of said protein in said
second antigen presenting cell is effected by a second
expression system;

(c) contacting the cells selected in step (b)
with a third antigen presenting cell, wherein the
expression of said protein in said third antigen
presenting cell is effected by a third expression system;
and

(d) testing the cells in step (c) with a fourth
antigen presenting cell and selecting for responding CTL
cells, wherein the expression of said protein in said
fourth antigen presenting cell is effected by a fourth
expression system;

wherein each said antigen presenting cell is an
autologous antigen presenting cell, and each of the
expression systems is different from at least one of the

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26    other expression systems, thereby isolating a cytotoxic T

27    cell clone specific for said protein.


1    2.      The method of claim 1, wherein said first,

2    second, third or fourth expression system is selected

3    from recombinant *Yersinia*, recombinant *Salmonella*,

4    recombinant ALVAC, recombinant vaccinia, recombinant

5    adenovirus, or recombinant retrovirus.


1    3.      The method of claim 1, wherein each said

2    antigen presenting cell is selected from the group

3    consisting of a macrophage, dendritic cell, a B cell, a

4    monocyte, marginal zone Kupffer cell, a Langerhans' cell,

5    an interdigitating dendritic cell, a follicular dendritic

6    cell, a T cell, an activated T cell, an astrocyte, a

7    follicular cell, an endothelium cell and a fibroblast.


1    4.      The method of claim 1, wherein said protein is

2    selected from the group consisting of members of the MAGE

3    family, the BAGE family, the DAGE/PRAME family, the GAGE

4    family, the RAGE family, the SMAGE family, NAG,

5    Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125,

6    p21ras, p53, HPV16 E7, the SSX family, HOM-MEL-55, NY-

7    COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11,

8    HOM-MEL-2.4, HOM-TES-11, RCC-3.1.3, NY-ESO-1, and the SCP

9    family.


1    5.      The method of claim 1, wherein said testing is

2    conducted by an assay selected from an IFN-gamma

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3    secretion assay, a TNF production assay, a $^{51}$Cr release
4    assay, an ELI-spot assay or a tetramer staining assay.


1    6.        A method for identifying an antigenic peptide
2    epitope of a protein, comprising:

3        (a) obtaining a sample containing T cells and
4    contacting the sample with a first antigen presenting
5    cell, wherein the expression of said protein in said
6    first antigen presenting cell is effected by a first
7    expression system;

8        (b) testing cells in said sample with a second
9    antigen presenting cell and selecting for responding CTL
10    cells, wherein the expression of said protein in said
11    second antigen presenting cell is effected by a second
12    expression system;

13        (c) contacting the cells selected in step (b)
14    with a third antigen presenting cell, wherein the
15    expression of said protein in said third antigen
16    presenting cell is effected by a third expression system;
17    and

18        (d) testing the cells in step (c) with a fourth
19    antigen presenting cell and  selecting for responding CTL
20    cells, wherein the expression of said protein in said
21    fourth antigen presenting cell is effected by a fourth
22    expression system;

23        wherein the antigen presenting cells are
24    immunocompatible with each other, and each of the
25    expression systems is different from at least one of the
26    other expression systems, thereby isolating a cytotoxic T
27    cell clone specific for said protein; and

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28         (e) identifying an antigenic peptide epitope of
29  said protein by using the cytotoxic T cell clone isolated
30  in step (d).


1      7.      A CTL clone isolated by the method of claim 1.


1      8.      An isolated CTL clone specific for a
2  peptide/HLA complex, wherein said complex is selected
3  from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-
4  Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID
5  NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2,
6  EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO:
7  55)/HLA-B40, and RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.


1      9.      An isolated CTL clone, wherein said clone is
2  selected from the group consisting of LB1137 462/F3.2,
3  LB1801 456/H7.11, LB1137 H4.13, LB1118 466/D3.31, LB1801
4  456/H8.33, LB1841 526/F7.1 and LB1803 483/G8.4.


1      10.     An isolated antigenic peptide, wherein said
2  peptide is selected from the group consisting of
3  DPARYEFLW(SEQ ID NO: 42), GVYDGREHTV(SEQ ID NO: 44),
4  SAFPTTINF(SEQ ID NO: 47), EVYDGREHSA(SEQ ID NO: 48),
5  AELVHFLLL (SEQ ID NO: 55) and RVRFFFPSL (SEQ ID NO: 57).


1      11.     An isolated nucleic acid molecule encoding said
2  peptide of claim 10.

1    12.      An isolated peptide/HLA complex, wherein said
2    complex is selected from the group consisting of
3    SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO:
4    42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2,
5    SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO:
6    48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and
7    RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.


1    13.      An antigen presenting cell expressing said
2    peptide/HLA complex of claim 12.


1    14.      A pharmaceutical composition comprising a
2    pharmaceutical carrier and said CTL clone of claim 7.


1    15.      A pharmaceutical composition comprising a
2    pharmaceutical carrier and at least one isolated CTL
3    clone selected from the group consisting of a CTL clone
4    specific for SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, a CTL clone
5    specific for DPARYEFLW(SEQ ID NO: 42)/HLA-B53, a CTL
6    clone specific for GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, a
7    CTL clone specific for SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2,
8    a CTL clone specific for EVYDGREHSA(SEQ ID NO: 48)/HLA-
9    A28, a CTL clone specific for AELVHFLLL(SEQ ID NO:
10    55)/HLA-B40, and a CTL clone specific for RVRFFFPSL (SEQ
11    ID NO: 57)/HLA-B7.

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1    16.      A pharmaceutical composition comprising a
2    pharmaceutical carrier and at least one peptide selected
3    from the group consisting of DPARYEFLW(SEQ ID NO: 42),

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4       GVYDGREHTV(SEQ ID NO: 44), SAFPTTINF(SEQ ID NO: 47),

5       EVYDGREHSA(SEQ ID NO: 48), AELVHFLLL (SEQ ID NO: 55) and

6       RVRFFFPSL (SEQ ID NO: 57).

1       17.      A pharmaceutical composition comprising a

2       pharmaceutical carrier and at least one isolated

3       peptide/HLA complex selected from the group consisting of

4       SAYGEPRKL (SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO:

5       42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2,

6       SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO:

7       48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and

8       RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.

1       18.      A pharmaceutical composition comprising a

2       pharmaceutical carrier and at least one antigen

3       presenting cell selected from the group consisting of an

4       antigen presenting cell expressing SAYGEPRKL(SEQ ID NO:

5       2)/HLA-Cw3, an antigen presenting cell expressing

6       DPARYEFLW(SEQ ID NO: 42)/HLA-B53, an antigen presenting

7       cell expressing GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, an

8       antigen presenting cell expressing SAFPTTINF(SEQ ID NO:

9       47)/HLA-Cw2, an antigen presenting cell expressing

10      EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, an antigen presenting

11      cell expressing AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and an

12      antigen presenting cell expressing RVRFFFPSL (SEQ ID NO:

13      57)/HLA-B7.

1       19.      A method of detecting in a sample from a

2       subject, the presence of a cell which abnormally

3       expresses a peptide/HLA complex at the cell surface,

4  wherein said peptide/HLA complex is any one of
5  SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO:
6  42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2,
7  SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO:
8  48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, or
9  RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising
10 contacting said sample with a CTL clone specific for said
11 complex.


1   20.      A method of diagnosing in a subject, a
2   pathological condition characterized by an abnormal
3   expression of a peptide/HLA complex, wherein said
4   peptide/HLA complex is selected from the group consisting
5   of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO:
6   42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2,
7   SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO:
8   48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and
9   RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising
10  detecting in the subject, the presence of cells which
11  abnormally express said peptide/HLA complex at the cell
12  surface.


1   21.      A method of detecting in a sample of a subject,
2   the presence of CTL cells specific for a peptide/HLA
3   complex, wherein said complex is selected from the group
4   consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3,
5   DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO:
6   44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2,
7   EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO:
8   55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said
9   method comprising contacting said sample with a cell

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10    which presents said peptide/HLA complex on the surface of
11    said cell, and detecting a CTL response as an indication
12    of the presence of said CTL cells.


1     22.      A method of diagnosing in a subject, a
2     pathological condition characterized by an abnormal
3     expression of a peptide/HLA complex, wherein said
4     peptide/HLA complex is selected from the group consisting
5     of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO:
6     42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2,
7     SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO:
8     48)/HLA-A28,_AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and
9     RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising
10    detecting in the subject, an increased frequency of CTL
11    cells specific for said complex.


1     23.      A method of treating a subject having a
2     pathological condition characterized by abnormal
3     expression of a peptide/HLA complex, wherein said complex
4     is selected from the group consisting of SAYGEPRKL(SEQ ID
5     NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53,
6     GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO:
7     47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28,
8     AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID
9     NO: 57)/HLA-B7, comprising administering to said subject,
10    a therapeutically effective amount of CTL cells specific
11    for said complex.


1     24.      A method of treating a subject having a
2     pathological condition characterized by abnormal

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3   expression of a peptide/HLA complex, wherein said complex
4   is selected from the group consisting of SAYGEPRKL(SEQ ID
5   NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53,
6   GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO:
7   47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28,
8   AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID
9   NO: 57)/HLA-B7, comprising administering to said subject,
10  a therapeutically effective amount of the peptide in said
11  peptide/HLA complex.


1   25.      A method of treating a subject having a
2   pathological condition characterized by abnormal
3   expression of a peptide/HLA complex, wherein said complex
4   is selected from the group consisting of SAYGEPRKL(SEQ ID
5   NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53,
6   GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO:
7   47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28,
8   AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID
9   NO: 57)/HLA-B7, comprising administering to said subject,
10  a therapeutically effective amount of cells which present
11  said complex on the cell surface.


1   26.      The method of claim 25, wherein said cells are
2   autologous antigen presenting cells and wherein the
3   presentation of said said peptide/HLA complex on the cell
4   surface is achieved by loading said cells with the
5   peptide in said peptide/HLA complex.


1   27.      The method of claim 25, wherein said cells are
2   autologous antigen presenting cells and wherein the

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3    presentation of said peptide/HLA complex on the cell
4    surface is achieved by transfecting said cells with a
5    nucleotide sequence coding for the peptide in said
6    peptide/HLA complex.

# FIG. 1
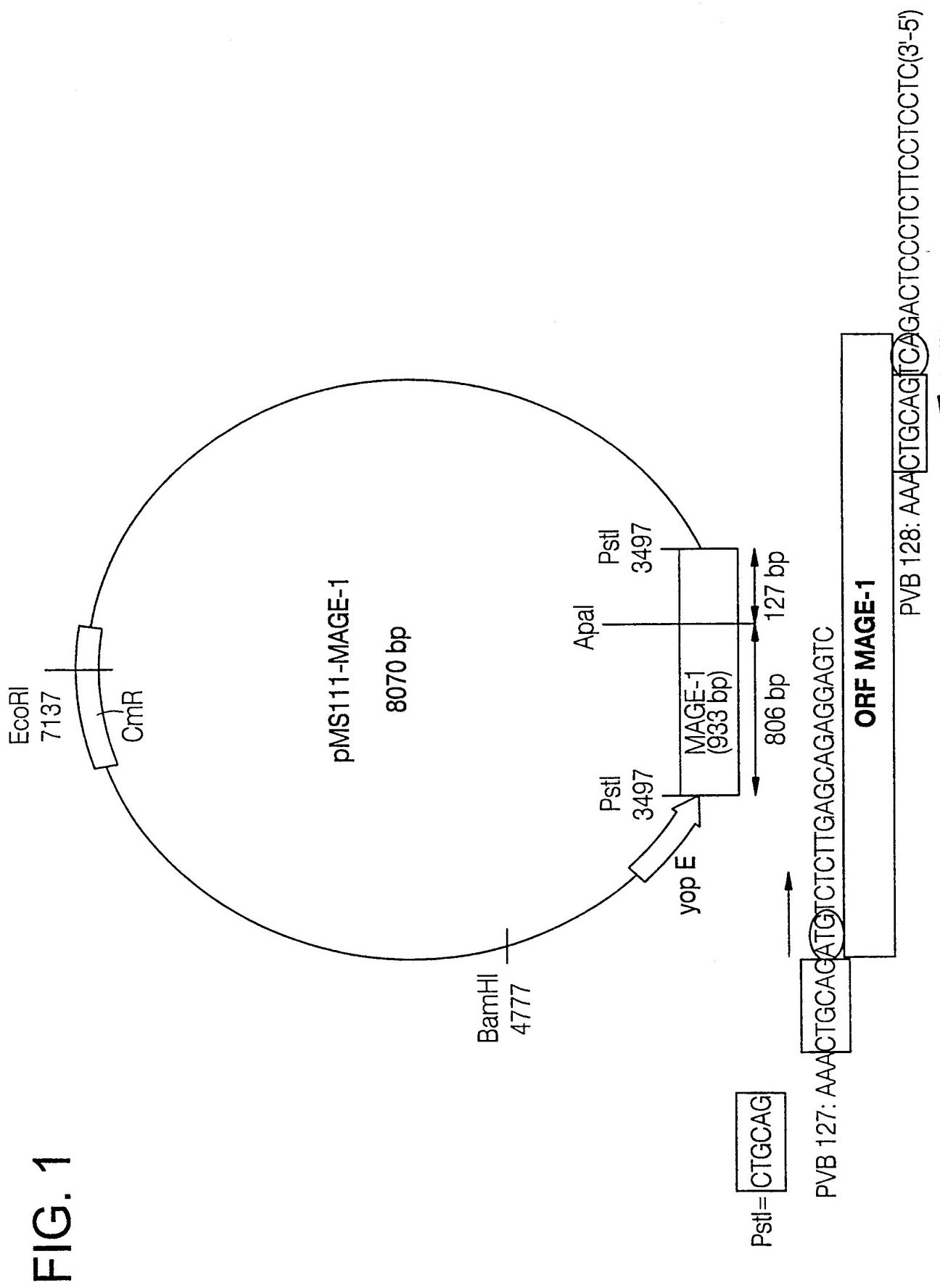
FIG. 2A
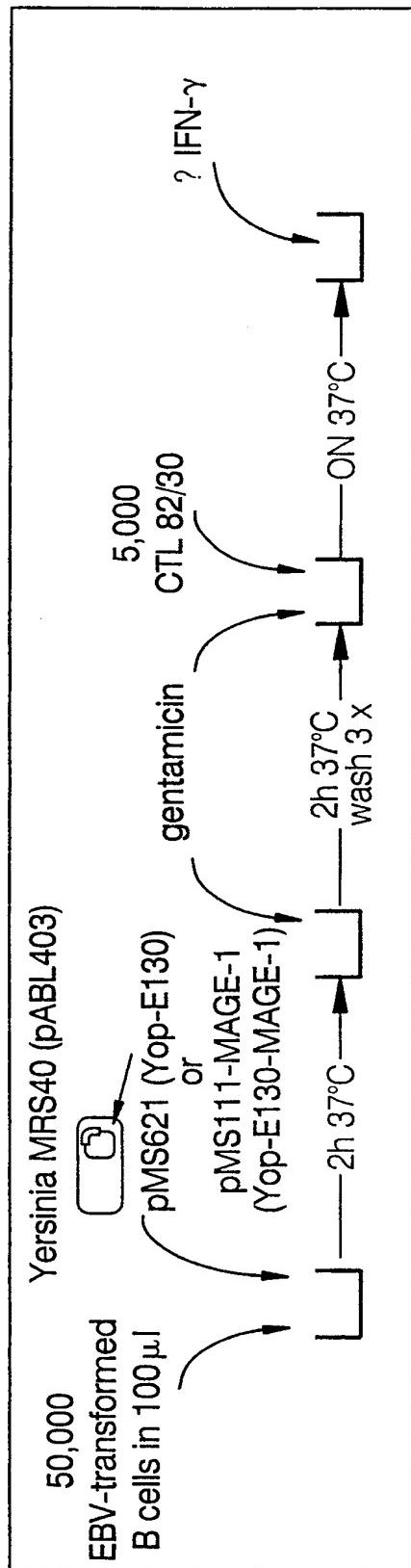
# FIG. 2B
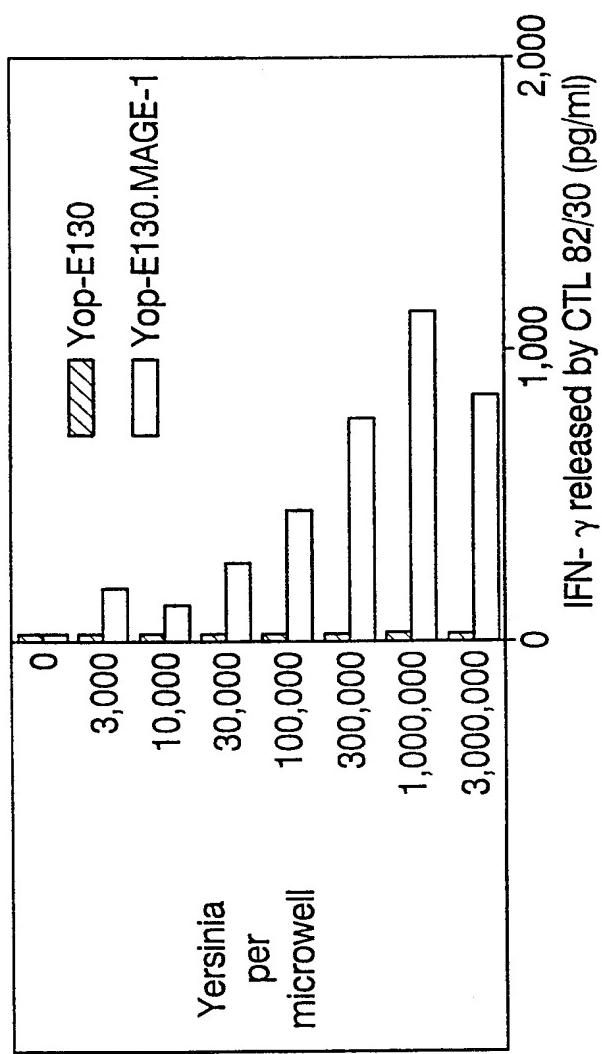
A MAGE-1 antigenic peptide is presented by HLA-Cw3 to CTL clone F3/2



FIGURE 3A
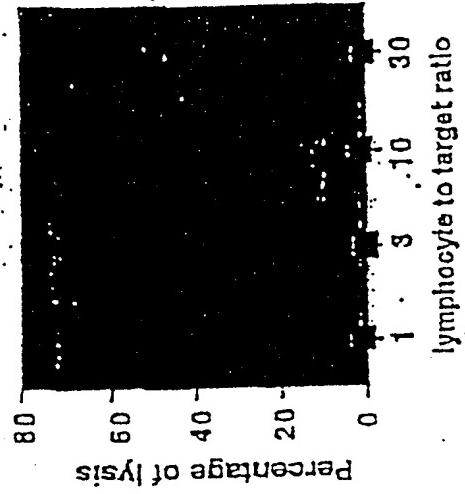
FIGURE 3B
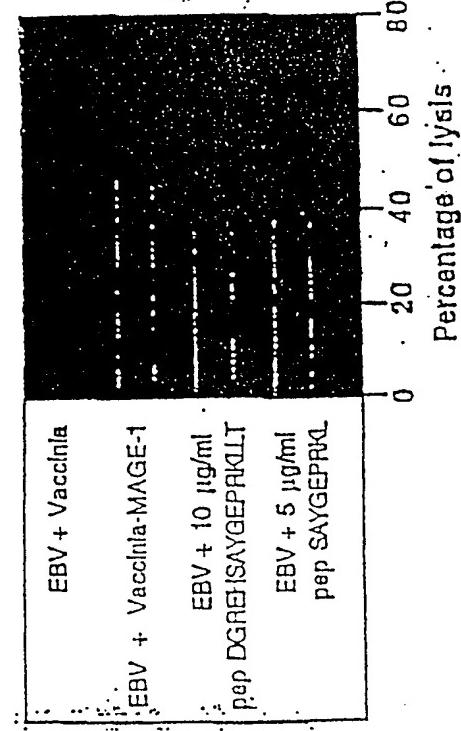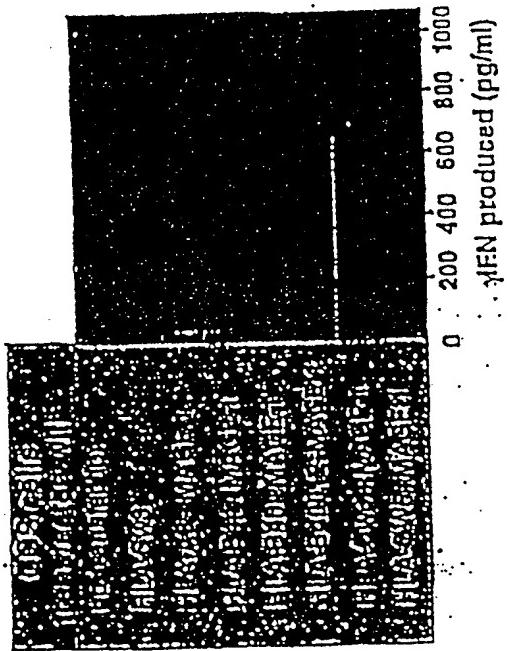
FIGURE 3C
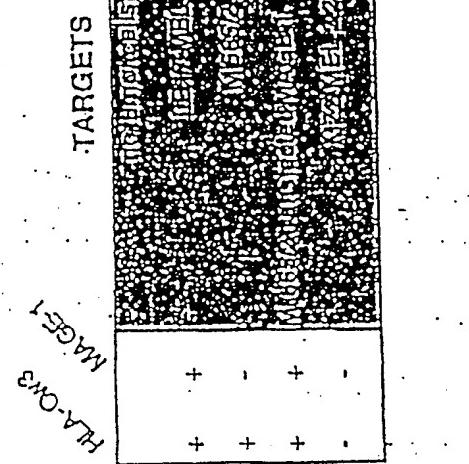
FIGURE 3D
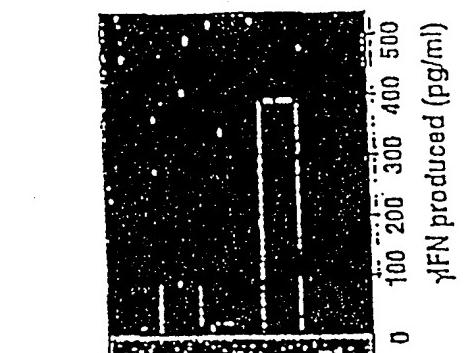
FIGURE 3

A MAGE-1 antigenic peptide is presented by HLA-B53 to CTL clone 456/H7.11

FIGURE 4B
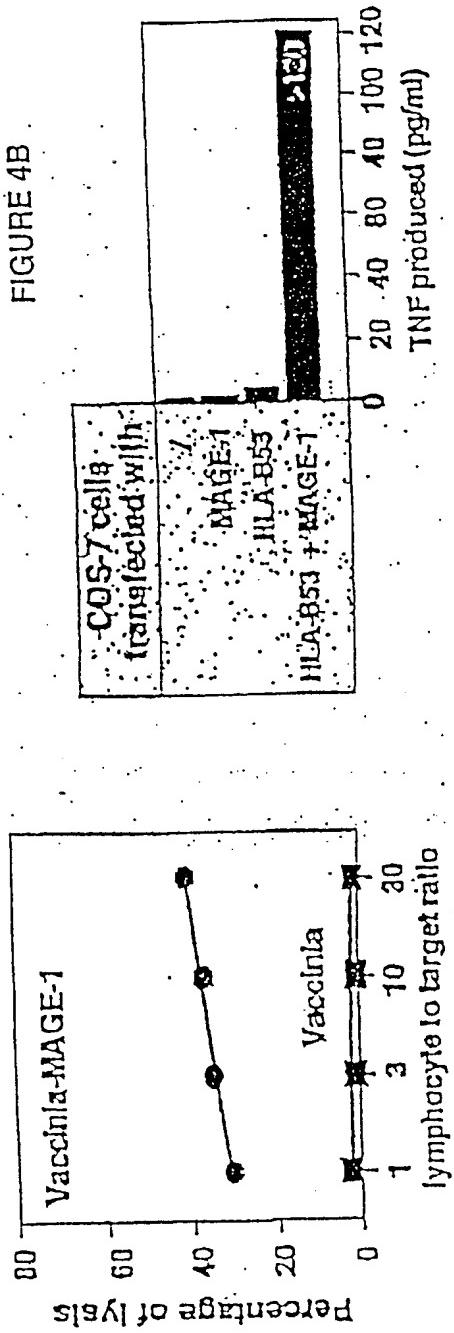
FIGURE 4A
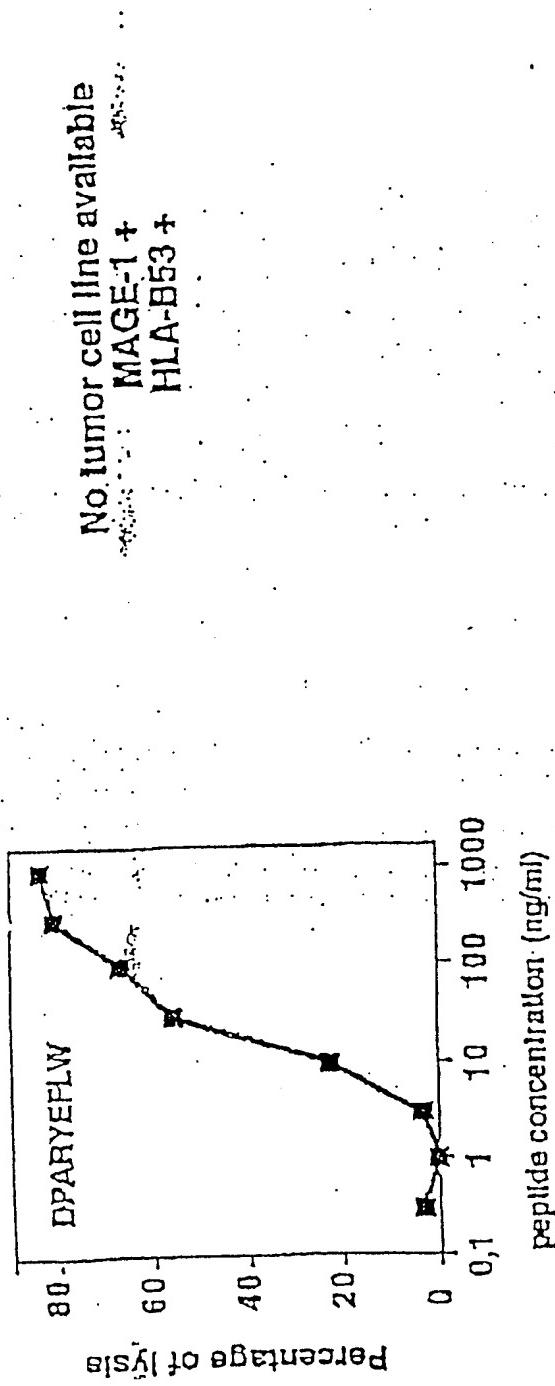
FIGURE 4C

FIGURE 4

Figure 5

A MAGE-4 antigenic peptide is presented by HLA-A2 to CTL clone H4/13
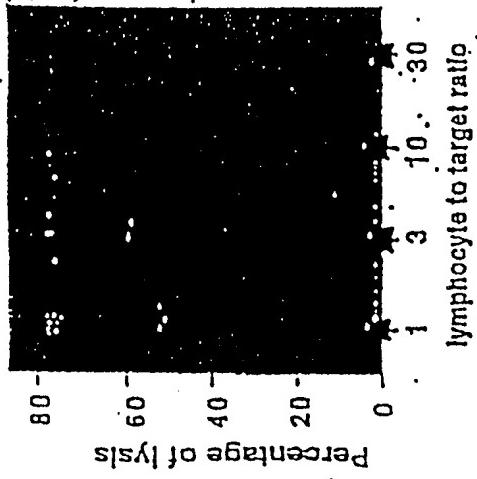


FIGURE 5A
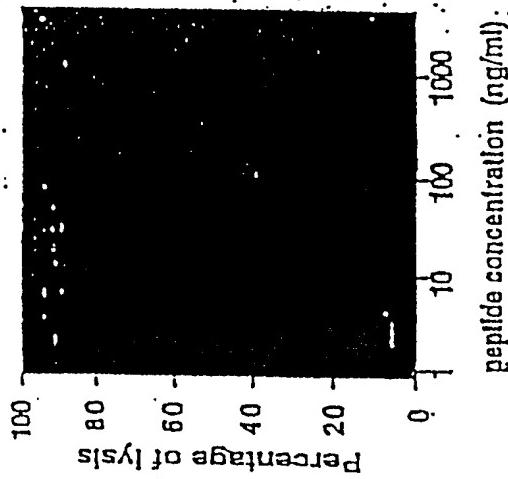
FIGURE 5B
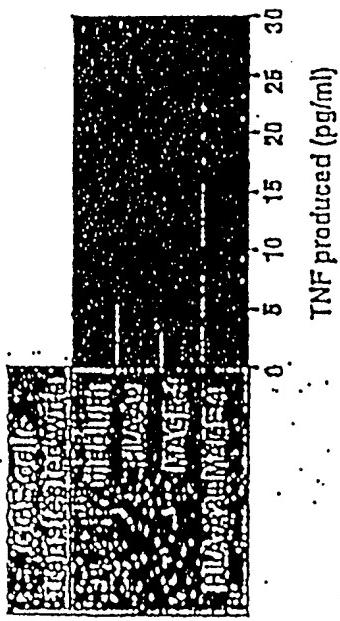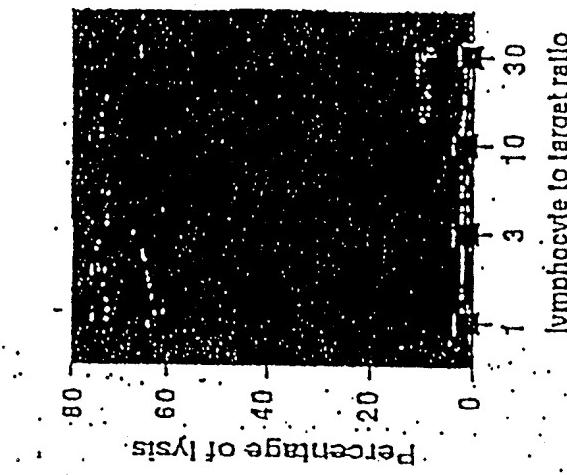
FIGURE 5C

FIGURE 5D
1998-PvdB 21 (M4A2 CTL)

# FIG. 6

AGTCATCATGTCTTCTGAGCAGAAGAGTCAGCACTGCAAGCCTGAGGA
                          S

AGGCGTTGAGGCCCAAGAAGAGGCCCTGGGCCTGGTGGGTGCACAGGC
                          AS8

TCCTACTACTGAGGAGCAGGAGGCTGCTGTCTCCTCCTCCTCTCCTCTG

GTCCCTGGCACCCTGGAGGAAGTGCCTGCTGCTGAGTCAGCAGGTCCTC
                          AS7

CCCAGAGTCCTCAGGGAGCCTCTGCCTTACCCACTACCATCAGCTTCA

CTTGCTGGAGGCAACCCAATGAGGGTTCCAGCAGCCAAGAAGAGGAGG

GGCCAAGCACCTCGCCTGACGCAGAGTCCTTGTTCCGAGAAGCACTCA
                          AS6

GTAACAAGGTGGATGAGTTGGCTCATTTTCTGCTCCGCAAGTATCGAG

CCAAGGAGCTGGTCACAAAGGCAGAAATGCTGGAGAGAGTCATCAAA

AATTACAAGCGCTGCTTTCCTGTGATCTTCGGCAAAGCCTCCGAGTCC
        AS5

CTGAAGATGATCTTTGGCATTGACGTGAAGGAAGTGGACCCCGCCAGC

AACACCTACACCCTTGTCACCTGCCTGGGCCTTTCCTATGATGGCCTG
                          AS4

CTGGGTAATAATCAGATCTTTCCCAAGACAGGCCTTCTGATAATCGTC

CTGGGCACAATTGCAATGGAGGGGCGACAGCGCCTCTGAGGAGGAAATC

TGGGAGGAGCTGGGTGTGATGGGGGTGTATGATGGGAGGGAGCACACT
                          AS3

GTCTATGGGGAGCCCAGGAAACTGCTCACCCAAGATTGGGTGCAGGAA

AACTACCTGGAGTACCGGCAGGTACCCGGCAGTAATCCTGCGCGCTAT
                                          AS2

GAGTTCCTGTGGGGTCCAAGGGCTCTGGCTGAAACCAGCTATGTGAAA
        AS2

GTCCTGGAGCATGTGGTCAGGGTCAATGCAAGAGTTCGCATTGCCTAC

CCATCCCTGCGTGAAGCAGCTTTGTTAGAGGAGGAAGAGGGAGTCTGA
                          AS1

**SUBSTITUTE SHEET (RULE 26)**

# FIG. 7A



# FIG. 7B



# FIG. 7C



# FIG. 7D

# FIG. 8A



- ● vaccinia-MAGE-A1
- ○ vaccinia control

% specific lysis

effector to target ratio

# FIG. 8B



Stimulator cells :
COS-7 cells
transfected with

| | |
|---|---|
| HLA-A2 + MAGE-1 | |
| HLA-A28 + MAGE-1 | > 120 |
| HLA-B4402 + MAGE-1 | |
| HLA-B53 + MAGE-1 | |
| HLA-Cw4 + MAGE-1 | |
| HLA-Cw5 + MAGE-1 | |

TNF release (pg/ml)

# FIG. 8C



% specific lysis

peptide EVYDGREHSA (nM)

# FIG. 8D



% specific lysis

effector to target ratio

| | HLA-A28 target cells | MAGE-A1 expression |
|---|---|---|
| ● LB 33-MEL.1C⁻ cl 3 | | + |

# FIG.9A



# FIG.9B

| Stimulator COS-7 cells transfected with | TNF (pg/ml) |
|---|---|
| MAGE-A3 | |
| HLA-B40 | |
| MAGE-A3+HLA-B40 | |
| MAGE-A3+HLA-B35 | |
| MAGE-A3+ HLA-A3 | |
| MAGE-A3+HLA-Cw3 | |
| MAGE-A3+HLA-Cw4 | |

# FIG.9C



# FIG.9D

# FIG.10A



# FIG.10B

# FIG.10C



# FIG.10D